PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
A61K 38/19, 38/27, C07K 14/475, 14/52, 14/61, 14/71, 14/715, 14/72

A1

(11) International Publication Number: **WO 99/58142**

(43) International Publication Date: 18 November 1999 (18.11.99)

(21) International Application Number: PCT/US99/10232

(22) International Filing Date: 11 May 1999 (11.05.99)

(30) Priority Data:

60/085,128	12 May 1998 (12.05.98)	US
09/246,041	5 February 1999 (05.02.99)	US

(71)(72) Applicants and Inventors: CHEN, Wen, Y. [CN/US]; 107 E. Brookwood Drive, Clemson, SC 29631 (US). WAGNER, Thomas, E. [US/US]; 104 Golden Wings Way, Greer, SC 29650 (US).

(74) Agents: KOLE, Lisa, B. et al.; Baker & Botts, LLP, 30 Rockefeller Plaza, New York, NY 10112-0228 (US).

(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: USE OF ANTI-PROLACTIN AGENTS TO TREAT PROLIFERATIVE CONDITIONS

(57) Abstract

The present invention relates to variant forms of human prolactin which act as antagonists at the prolactin receptor, and to the use of such variants in the treatment of human cancers and proliferative disorders, including both begnin and malignant diseases of the breast and prostate.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

USE OF ANTI-PROLACTIN AGENTS TO TREAT PROLIFERATIVE CONDITIONS

SPECIFICATION

1. INTRODUCTION

The present invention relates to methods and compositions for inhibiting the cell proliferation-promoting effects of prolactin on its receptor. The methods and compositions of the invention may be used in the treatment of benign as well as malignant conditions which involve unwanted cell proliferation.

2. BACKGROUND OF THE INVENTION

Prolactin ("PRL") is a 23-kDa neuroendocrine hormone which is structurally related to growth hormone and, to a lesser degree, to members of the interleukin family (Reynolds et al., 1997, Endocrinol. 138:5555-5560, Cunningham et al., 1990, Science 247:1461-1465; Wells et al., 1993, Recent Prog. Horm. Res. 48:253-275). Acting via the prolactin receptor, it is required for the proliferation and terminal differentiation of breast tissue (Mani et al., 1986, Cancer Res. 46:1669-1672; Malarkey et al., 1983, J. Clin. Endocrinol. Metab. 56:673-677; Biswas and Vonderhaar, 1987, Cancer Res. 47:3509-3514), promoting the growth and differentiation of the ductal epithelium, proliferation and differentiation of lobular units, and initiation and maintenance of lactation (Kelly et al., 1993, Recent Prog. Horm. Res. 48:123-164; Shiu et al., 1987, recent Pro. Horm. Res. 43:277-303). A diversity of other effects have been attributed to PRL, including roles in reproduction and the immune response (Wennbo et al., 1997, Endocrinol. 138:4410-4415; Nicoll, 1974, in *Handbook of Physiology*, Knobil and Sawyer, eds., American Physiological Society, Washington, D.C.; Shiu and Friesen, 1980, Annu. Rev. Physiol. 42:83-96).

The prolactin receptor ("PRLR") is a member of the cytokine receptor superfamily and binds a group of hormones, including not only PRL but also placental lactogens and primate growth hormone ("GH"), to produce a mitogenic effect

(Ormandy et al., 1997, J. Clin. Endocrinol. Metab. 82:3692-3699; Horseman, 1995, Endocrinol. 136:5249-5251; Clevenger et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:6460-6464; Buckley et al., 1985, Life Sci. 37:2569-2575; Costello et al., 1994, Prostate 24:162-166). PRLR is homologous to the receptor for GH ("GHR", also referred to as the somatogen receptor) and both belong to the cytokine receptor superfamily (Kelly et al., 1991, Endocrin. Rev. 12:235-251; Kelly et al., 1993, Recent. Prog. Horm. Res. 48:123-164; Horseman and Yu-Lee, 1994, Endocrin. Rev. 15:627-649).

An association between PRL activity and breast cancer has been proposed (Ormandy et al., 1997, J. Clin. Endocrinol. Metab. 82:3692-3699). Elevated PRL levels have been found to accelerate the growth of mammary tumors induced by 7,12 dimethylbenz(α)antracene in rats, whereas PRL ablation was observed to have an inhibitory effect (Welsch, 1985, Cancer Res. 45:3415-3443). Mammary tumor growth was increased in transgenic mice overexpressing human GH, which binds to the rodent PRLR (Bartke et al., 1994, Proc. Soc. Exp. Biol. Med. 206:345-359). It has been found that the receptors for sex steroids and PRL are co-expressed and cross-regulated, which might explain the synergistic actions of estrogen, progesterone, and PRL in tumor growth control (Ormandy et al., 1997, J. Clin. Endocrinol. Metab. 82:3692-3699).

Nevertheless, to date, therapies which reduce PRL levels, such as hypophysectomy and bromocriptine administration (both directed toward decreasing or eliminating production of PRL by the pituitary gland), have not been successful in the treatment of breast cancer (Peyrat et al., 1984, Eur. J. Cancer Clin. Oncol. 20:1363-1367; Heuson et al., 1972, Eur. J. Cancer 8:155-156). It has been proposed that PRL may nevertheless have a role in breast cancer if an autocrine/paracrine growth regulatory loop exists (that is to say, that the pituitary is only one of several sources for prolactin; see Clevenger et al., 1995, Am. J. Pathol. 146:695-705, Fields et al., 1993, Lab. Invest. 68:354-360; Ginsburg and Vonderhaar, 1995, Cancer Res. 55:2591-2595; Fuh and Wells, 1995, J. Biol. Chem. 270:13133-13137). In this regard, when RNA levels of PRL and PRLR were performed using reverse transcriptase/PCR techniques, it was found that PRL and PRLR were widely

expressed in breast cancers (>95 percent) and normal breast tissues (>93 percent), suggesting that interventions in the PRL/PRLR receptor may be useful in the treatment of breast cancer (Reynolds et al., Endocrinol. 138:5555-5560). Indeed, it has recently been reported that a combined regimen combining an anti-estrogen (tamoxifen), a GH analog (octreotide), and a potent anti-prolactin (CV 205-502, a dopamine agonist which inhibits prolactin secretion by the pituitary) had better clinical results in metastatic breast cancer patients compared to tamoxifen therapy alone (Botenbal et al., 1998, Br. J. Cancer 77:115-122).

An association between PRL expression and prostate disease has also been proposed (Wennbo et al., 1997, Endocrinol. 138:4410-4415). PRL receptors are found in prostate tissue (Aragona and Friesen, 1975, Endocrinol. 97:677-684; Leake et al., 1983, J. Endocrinol. 99:321-328). PRL levels have been observed to increase with age (Hammond et al., 1977, Clin. Endocrinol. 7:129-135; Vekemans and Robyn, 1975, Br. Med. J. 4:738-739) coincident with the development of prostate hyperplasia and PRL has been found to have trophic and differentiating effects on prostate tissue (Costello and Franklin, 1994, Prostate 24:162-166). Transgenic mice overexpressing the PRL gene developed dramatic enlargement of the prostate gland (Wennbo et al., 1997, Endocrinol. 138:4410-4415). Nonetheless, the role for PRL in prostate disease remains unclear (Wennbo et al., 1997, Endocrinol. 138:4410-4415). PRL levels in patients having prostate hyperplasia have been reported to be either increased (Odoma et al., 1985, J. Urol. 133:717-720; Saroff et al., 1980, Oncology 37:46-52), increased only in patients with prostate cancer or unchanged (Harper et al., 1976, Acta Endocrinol. (Copenh) 81:409-426). Janssen et al. reported that proliferation of androgen-insensitive human prostate cell lines can be significantly modulated by PRL (1996, Cancer 77:144-149). To explain these discrepancies, it has been proposed that local synthesis of PRL in the prostate (Nevalainen et al., 1997, J. Clin. Invest. 99:618-627) may be an important factor. Androgen-dependent expression of PRL in rat prostate epithelium has been observed, supporting the concept of an autocrine/paracrine loop of prolactin action in the prostate, where it could mediate androgen-associated effects (Nevalainen et al., 1997, FASEB J. 11(14):1297-1307). Further, clinical data appears promising: hypophysectomy has been found to have an

additive therapeutic effect when combined with castration and adrenalectomy in prostate cancer patients (Brendler, 1973, Urology 2:99-102), and Rana et al. report that a combined maximal suppression of androgens and prolactin resulted in a significantly improved clinical response over conventional treatments in patients suffering from advanced prostate cancer (Habib et al., 1995, Eur. J. Cancer 31A:859-860).

In view of the biological relevance of the PRL molecule and its receptor, a number of investigators have evaluated the activity of PRL variants which bear structural differences relative to the native unmodified molecule. It has been reported that naturally phosphorylated rat PRL antagonizes the growth-promoting effects of unmodified PRL in an assay which measures proliferation of rat Nb2 T lymphoma cells and in the autocrine regulation of GH_3 cell proliferation (Wang and Walker, 1993, Endocrinol. 133:2156-2160; Krown et al., 1992, Endocrinol. 122:223-229). Further, molecular mimics of phosphorylated PRL having a bulky negatively charged amino acid (namely glutamate or aspartate) substituted for the serine at position 179 antagonized the growth-promoting effects of PRL (Chen et al., 1998, Endocrinol. 139: 609-616).

Other strategies for PRL variant design have been directed at disruption of the interaction between PRL and its receptor. To this end, researchers have drawn analogies between the PRLR and the GHR, for which the structure/function relationships are better understood.

Certain features of the GHR were elucidated by studying the basis for the full GH antagonist activity of the variant of human GH ("hGH") having a substitution of the glycine at position 120 with an arginine residue (Chen et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:5061-5065; Chen et al., 1991, Mol. Endocrinol. 5:1845-1852; Chen et al., 1994, J. Biol. Chem. 269:15892-15897; Chen et al., 1995, Mol. Endocrinol. 9:1-7; United States Patent No. 5,350,836 by Kopchick and Chen; United States Patent No. 5,681,809 by Kopchick and Chen). It was deduced that hGH forms a complex with a dimeric form of the hGHR. Fuh and colleagues proposed a sequential dimerization model whereby GH would first bind to one receptor via a first binding site (delimited by portions of helix 1, helix 4 and loop 1 of GH) to form an

inactive intermediate 1:1 complex, and then the receptor-bound hGH would interact with a second receptor through binding site 2 (involving the helix 3 glycine of GH mutated in the G120R variant) to produce the active 1:2 hormone/receptor complex (Fuh et al., 1992, Science 256:1677-1680; Fuh et al., 1993, J. Biol. Chem. 268:5376-5381, Goffin et al., 1994, J. Biol. Chem. 269:32598-32606). When the helix 3 glycine at position 120 of GH is substituted with an arginine residue, the second binding site is sterically hindered and the GH can no longer induce receptor dimerization.

Although less is known about the structure of the PRLR, it has been suggested that it, too, is activated by hormone-mediated sequential dimerization (Cunningham et al., 1991, Proc. Natl. Acad. Sci. U.S.A. 88:3407-3411; Fuh et al., 1992, Science 256: 1677-1680; Fuh et al., 1993, J. Biol. Chem. 268:5376-5381). Variants of human PRL ("hPRL") were produced containing mutations in the region believed to correspond to the helix 3/helix 1 interface of GH, including mutations of the alanine at position 22, the leucine at position 25, the serine at position 26 and the glycine at position 129 of PRL to tryptophan and/or arginine (specifically, to create A22W, L25R, L25W, S26R, S26W and G129R; Goffin et al., 1994, J. Biol. Chem. 269:32598-32606). It was reported in that paper that the point mutations at A22, S26 and G129 drastically decreased the mitogenic potency of the variant (as compared to native PRL) by 2-3 orders of magnitude (as tested in the Nb2 proliferation assay), although the G129R variant (positionally analogous to G120R of GH) was reported to act as a weak agonist rather than as an antagonist. It was subsequently reported that when tested in an assay for PRLR activity in which cells, co-transfected with nucleic acid encoding the hPRLR and a reporter gene under the control of PRL-responsive DNA sequences, were exposed to the G129R hPRL variant, an antagonist effect was observed (Goffin et al., 1996, J. Biol. Chem. 271:16573-16579).

Naturally occurring antagonists of GH action may exist. A cell-free truncated form of the GHR (termed "GH-BP") has been identified in man and certain animals (Baumann, 1991, Acta Endocrinol. 124(suppl 2):21-26; Baumann et al., 1994, J. Endocrinol. 141:1-6; Baumann, 1995, Endocrinol. 136:377-378). The human form of GH-BP encompasses the extracellular domain of the receptor, and could be the result of proteolytic cleavage of the native receptor or alternative RNA splicing. It has

been suggested that GH-BP acts to inhibit binding of GH to its receptors (Baumann, 1991, Acta Endocrinol. 124(suppl 2):21-26; Baumann et al., 1994, J. Endocrinol. 141:1-6). Supportive of this hypothesis is the observation that GH-BP levels in patients suffering from acromegaly (due to overexpression of GH) have an inverse correlation with serum GH levels (that is to say, the less GH-BP, the more serum GH present; Amit et al., 1992, Hormone Res. 37:205-211). Lower levels of GH-BP may render the acromegalic serum GH relatively more active in the GH receptor assay and therefore contribute negatively to the disease (Hochberg et al., 1994, Acta Endocrinol. 125:23-27). Soluble forms of other receptors in the cytokine receptor superfamily have also been observed (Baumann, 1995, Endocrinol. 136:377-378). Nevertheless, there has not been, prior to the present invention, any evidence suggesting the existence of a naturally occurring cell-free from of the PRLR.

3. SUMMARY OF THE INVENTION

The present invention relates to methods and compositions for inhibiting the cell proliferation-promoting effects of prolactin on its receptor.

In a first set of embodiments, the present invention provides for a prolactin variant which acts as an antagonist at the prolactin receptor, and for the use of such a prolactin variant in inhibiting the proliferation of a cell which expresses a prolactin receptor. The invention is based in the observation that a prolactin variant is capable of inhibiting cell proliferation in a dose dependent manner. Further, it was observed that the prolactin variant was able to induce apoptosis in cancer cells. In preferred embodiments, the prolactin variant is a mutated form of human prolactin in which the glycine amino acid at position 129 is substituted with another amino acid. In specific nonlimiting embodiments, the glycine at position 129 of human prolactin is substituted with arginine.

In a second set of embodiments, the present invention provides for a truncated form of the prolactin receptor which is capable of binding to prolactin and thereby decreases the availability of prolactin to bind to its receptor. The prolactin variants and truncated prolactin receptors of the invention may be used in methods of inhibiting the proliferation of cells expressing prolactin receptors.

The present invention further provides methods for inducing apoptosis in cells expressing the prolactin receptor. The invention is based on the observation that a prolactin variant is capable of inducing cellular apoptosis in human breast cancer cells.

In yet another embodiment of the invention, the present invention provides methods of inhibiting the proliferation of cells expressing prolactin receptor comprising the use of a prolactin variant in conjunction with an anti-estrogen. Such anti-estrogens include, but are not limited to, tamoxifen, raloxifene, or ICI 164384 (Imperial Chemical Industries). The method is based on the observation that the administration of a prolactin variant together with an anti-estrogen induces a synergistic inhibitory effect on cell proliferation. In addition, a prolactin variant may be used in conjunction with an anti-androgen. Such anti-androgens include ,but are not limited to, flutamide, anandron or cyproterone acetate to induce a synergistic inhibition of cellular proliferation (see, Smith, D.C., 1997, Semin. Urol. Oncol. 15:3-12 for review of anti-androgen therapy; Gomella, I. M., 1997, 3:16-24; Suciu, S., 1993, Cancer 15:3841-6), .

Accordingly, such methods may be used in the treatment of clinical disorders in humans and non-human animals which involve unwanted cell proliferation. In specific nonlimiting embodiments, the present invention may be used in the treatment of breast and prostate cancers in humans.

4. BRIEF DESCRIPTION OF THE DRAWINGS

FIGURE 1A. Schematic representation of the cloning and construction of the expression plasmid pUCIG-MT-hPRLcDNA.

FIGURE 1B. Plasmid map and general strategy of PCR-directed mutagenesis. pcDNA3, the parental vector, contains human immediate-early cytomegalovirus (CMV) transcriptional regulatory sequences and a polyadenylation signal and transcription termination sequence from bovine GH gene (BGH pA). hPRL cDNA was cloned using RT-PCR from human pituitary mRNA and inserted into BstX1 sites. Mutation was generated by designing PCR primers at Xba I sites.

FIGURE 2. Data from competitive radioreceptor binding experiments for hGH and hPRL using various human cancer cell lines (listed along the x-axis). HTB123 and T47D are human breast cancer cell lines. The y axis represents the percent specific binding. Each point represents the mean of three experiments which were each carried out in duplicate.

FIGURE 3. Western blot analysis showing phosphorylation of STAT proteins (band at arrow) in T47D human breast cancer cells under various conditions. Reading from left to right, lane 1 depicts a control culture, lane 2 depicts a culture receiving 5 nM of hPRL, lane 3 depicts a culture receiving 5 nM of hPRLA, lane 4 depicts the competitive effects when the culture is exposed to 5 nM of hPRL and 5 nM of hPRLA, and lane 5 depicts the competitive effects when the culture is exposed to 5 nM hPRL and 25 nM hPRLA.

FIGURE 4. Effects of growth hormone and prolactin on breast cancer cell proliferation. The x-axis represents the concentration of hGH or hPRL present in the culture media of T-47D human breast cancer cells. The y axis represents the total cell number at the end of the incubation period. Points are the average (+SD) of three experiments, each of which was carried out in duplicate.

FIGURE 5A-B. (A) Effects of various concentrations of hPRL or the G129R prolactin variant hPRLA on the proliferation of T47D human breast cancer cells in culture. (B) Effects of various concentrations of estrogen (E2) and tamoxifen (Tam) on the proliferation of T47D cells over a period of 24 or 72 hours.

FIGURE 6. Diagram of a mixed cell culture assay for evaluating the effects of recombinant hPRL and the G129R prolactin variant hPRLA on T47D cell proliferation.

FIGURE 7. Effects of recombinantly expressed hPRL (L-PRL) and the G129R prolactin variant hPRLA (L-PRLA) on T47D breast cancer cell proliferation in a mixed cell culture assay after 24 and 72 hours.

FIGURE 8. Effects of recombinantly expressed hPRL (L-PRL) and the G129R prolactin variant (L-PRLA) on T47D breast cancer cell proliferation in a mixed cell culture assay after one (D1), two (D2), three (D3) or five (D5) days.

FIGURE 9A-B. Proliferation of either (A) T47D human breast cancer cells or (B) MCF-7 human breast cancer cells in mixed cell culture assays with L cells which express recombinant G129 human prolactin variant hPRLA after three days in culture.

FIGURE 10A-B. Amino acid sequences of various human and non-human forms of prolactin.

FIGURE 11. Schematic illustration of the mechanism of GH or hPRL (ligand) antagonist. Four helical regions in the ligand (dotted ovals) are labeled as I, II, III and IV. Two membrane bond receptors (shaded ovals) are also shown in the figure. Arg represents the substitution mutation in the third α-helix resulting in hindering a second receptor to form a functional complex (from A to B).

FIGURE 12. Immunoblot analysis of hPRL-G129R gene expression by mouse L cells transfected with the pcDNA3 vector genetically engineered to encode the G129R variant. Lanes A-D represent samples containing purified hPRL (from NIH) as standards. Lanes E-H represent culture media from stably transfected mouse L cells.

FIGURE 13. Antagonistic effects of hPRL-G129R on tyrosine phosphorylation of STAT proteins induced by hPRL in human breast cancer cells (T47-D). Lane assignments are A, negative control; B, cells stimulated with 100 ng/ml hPRL; C, cells treated with 100 ng/ml of hPRL-G129R; D, cells treated with 100 ng/ml of hPRl and 100 ng/ml of hPRL-G129R; E, cells treated with 100 ng/ml of hPRL and 500 ng/ml of hPRL-G129R. Arrow indicates the position of 95 kDa proteins.

FIGURE 14A-E. Light microscopic examination of T47-D human breast cancer cells after single dose of 200 ng/ml hPRL (15B); 200 ng/ml of hPRL-G129R (15C); 200 nM of E2 (15D); or 200 nM of 4-OH-Tamoxifen (15E) treatment after 4-day incubation as compared to control (15A). x 200.

FIGURE 15. Dose-response effects of hPRL and its synergistic effects with E2 in T47-D human breast cancer cell proliferation assay. The x-axis represents the hPRL concentration either in the absence (open bars) or presence of E2. Each data point represents a mean of at least three independent experiments with triplicate wells. Bars, SD.

FIGURE 16A-B. Dose-response effects of 4-OH-Tamoxifen (17A) and hPRl-G129R (17B). The x-axis represents the concentration of 4-OH-Tamoxifen (17A) and hPRl-G129R (17B). Each data point represents a mean of at least three independent experiments with triplicate wells. Bars, SD.

FIGURE 17. Dose-response inhibitory effects of hPRL-G129R on hPRL induced T47-D cell proliferation. The x-axis represents the concentration of hPRL-G129R either in the absence of hPRL (open bars) and the presence of hPRL. Each data point represents a mean of at least three independent experiments with triplicate wells. Bars, SD.

FIGURE 18. Dose-response inhibitory effects of hPRL-G129R and its synergistic effects with 4-OH-Tamoxifen in T47-D human breast cancer cell proliferation assay. The x-axis represents the hPRL-G129R concentration either in the absence (open bars) or presence of 4-OH-Tamoxifen. Each data point represents a mean of at least three independent experiments with triplicate wells. Bars, SD.

FIGURE 19A-B. Dose-response inhibitory effects of hPR1-G129R in two human breast cancer cell lines using co-culture method. The x-axis represents the co-cultured L cell (control) or L-hPRL-G129R cell numbers. Each data point represents a mean of at least three independent experiments with triplicates wells. Bars, SD.

FIGURE 20A-F. Dose response of T-47D human breast cancer cells to hPRL-G129R after 24 hours treatment using TUNEL assay (panels A-F). Panel (G) and (H) shows results of competition between hPRL and hPRL-G129R at 1:1 ratio (125ng/ml hPRL+125ng/ml hPRL-G129R; panel G) and 1:4 ratio (125ng/ml hPRL-G129R+500ng/ml hPRL, panel H).

FIGURE 21A-E. Time course of T-47D human breast cancer cells responding to hPRL-G129R treatment (50ng/ml) using TUNEL assay.

FIGURE 22A-H. Response of multiple breast cancer cells to 4-OH-Tamoxifen treatment (1μM for 24 hours) using TUNEL assay. Labels C and T stand for control and treated cells, respectively.

FIGURE 23A-F. Response of multiple breast cancer cells to treatment with 250ng hPRL-G129R for 24 hours using TUNEL assay. Labels C and T stand for control and treated cells, respectively.

FIGURE 24. Induction of Caspase-3 by hPRL-G129R. The effect of hPRL-G129R on Caspase-3 activation in T-47D cells using an ApopAlert CPP32/ Caspase-3 assay kit (Clontech, Palo Alto, CA) is shown. T-47D cells were treated with 250 ng/ml of hPRL-G129R for 2h. The assay was performed in the presence of DEVD-CHO (caspase-3 inhibitor) to demonstrate that the Caspase-3 induction by hPRL-G129R is a specific event. The samples were in duplicate and each sample constituted about 2 million cells.

FIGURE 25. Response of two prostate cancer cells to treatment with 250ng hPRL-G129R for 24 hours using TUNEL assay.

5. DETAILED DESCRIPTION OF THE INVENTION

For purposes of clarity, and not by way of limitation, the detailed description of the invention is divided into the following subsections:

(i) prolactin variants;

(ii) truncated prolactin receptors; and

(iii) utility of the invention.

5.1. PROLACTIN VARIANTS

The present invention provides for prolactin (PRL) variants which antagonize the action of PRL at its receptor.

The term prolactin (PRL) refers herein to human and nonhuman animal forms of the hormone prolactin. Such prolactins include, but are not limited to, prolactins for which the amino acid sequences are set forth in FIGURE 10 (see also Cooke et al., 1981, J. Biol. Chem. 256:4007; Cooke et al., 1980, J. Biol. Chem. 225:6502; Kohmoto et al., 1984, Eur. J. Biochem. 138:227; Tsubokawa et al., 1985, Int. J. Peptide Protein Res. 25:442; Bondar et al., 1991, GenBank Accession No. #X63235; Sasavage et al., 1982, J. Biol. Chem. 257:678; Miller et al., 1980, Endocrinol. 107:851; Li et al., 1970, Arch. Biochem. Biophys. 141:705; Li, 1976, Int.

J. Peptide Protein Res. 8:205; Martinant et al., 1991, Biochim. Biophys. Acta 1077:339; Lehrman et al., 1988, Int. J. Peptide Protein Res. 31:544; Li et al., 1989, Int. J. Peptide Protein Res. 33:67; Hanks et al., 1989, J. Mol. Endocrinol. 2:21; Watahiki et al., 1989. J. Biol. Chem. 264:5535; Karatzas et al., 1990, Nucl. Acids Res. 18:3071; Yasuda et al., 1990, Gen. Comp. Endocrinol. 80:363; Noso et al., Int. J. Peptide Protein Res. 39:250; Buckbinder et al., 1993, Proc. Natl. Acad. Sci. U.S.A. 90:3820; Takahashi et al., J. Mol. Endocrinol. 5:281; Yamaguchi et al., 1988, J. Biol. Chem. 263:9113; Rentler-Delrue et al., DNA 8:261; Yasuda et al., 1987, Gen. Comp. Endocrinol. 66:280; Chang et al., 1991, GenBank Acc. No. #X61049; Chang et al., 1991, GenBank Acc. No. #X61052; Yasuda et al., 1986, Arch. Biochem. Biophys. 244:528; Kuwana et al., 1988, Agric. Biol. Chem. 52:1033; Song et al., 1988, Eur. J. Biochem. 172:279: Mercier et al., 1989, DNA 8:119).

The term prolactin (PRL) variant refers to a form of prolactin which has been structurally altered relative to its native form, including where the amino acid sequence of the native form has been altered by the insertion, deletion, and/or substitution of amino acids.

The ability of such a variant to antagonize the action of PRL at its receptor is defined as the ability of the variant to inhibit an effect mediated, under normal conditions, by PRL. For example, where PRL has a proliferative effect on a species of cell, a PRL variant according to the invention inhibits the proliferation of the species of cells: without being limited by the following theory, it is believed that PRL is present at some level for an inhibitory effect to be observed. FIGURE 5A illustrates a working example of the invention in which human prolactin (hPRL) induces the proliferation of T47D human breast cancer cells, whereas a variant of hPRL having a substitution of the glycine at position 129 with an arginine residue, termed hPRLA, inhibits proliferation of T47D cells relative to T47D cells lacking the added hPRL or hPRLA; it is believed that T47D levels produce PRL (Ginsberg and Vonderharr, 1995, Cancer Res. 55:2591-2595).

As a specific non-limiting example, a PRL variant may be identified as an antagonist of PRL by determining the ability of the variant to block the ability of PRL to act via its receptor when both PRL and the PRL variant are present. As an

example, where a given concentration X of PRL is associated with an increase Y in the proliferation of cells expressing the PRLR in culture, when a comparable sample of cells are exposed to PRL at concentration X, and a PRL variant at a concentration V, the proliferation of the cells will increase by Z, where Z is less than Y and may be a negative number.

In one non-limiting embodiment of the invention, the PRL variant is a variant of human PRL having a substitution of the glycine at position 129 with another amino acid. The substitution, represented in shorthand form by G129*, where * is a naturally occurring or synthetic amino acid other than glycine, may be the sole variation from the native sequence or one of several alterations (including insertions, deletions, and/or substitutions of amino acids). The substituent amino acid may be neutral-polar amino acids such as alanine, valine, leucine, isoleucine, phenylalanine, proline, methionine; neutral non-polar amino acids such as serine, threonine, tyrosine, cysteine, tryptophan, asparagine, glutamine, aspartic acid; acidic amino acids such as aspartic and glutamic acid; and basic amino acids such as arginine, histidine or lysine. In preferred embodiments of the invention, the glycine at position 129 of hPRL may be substituted with valine, leucine, isoleucine, serine, threonine, proline, tyrosine, cysteine, methionine, arginine, histidine, tryptophan, phenylalanine, lysine, asparagine, glutamine, aspartic acid, and glutamic acid. In a most preferred embodiment of the invention, the substitution replaces the glycine at position 129 with arginine (G129R). In a further specific nonlimiting embodiment, the present invention provides for a prolactin variant wherein the glycine at position 129 is deleted.

In yet other nonlimiting embodiments, a prolactin variant is linked to another protein as part of a fusion protein. As one specific embodiment, the prolactin variant may be linked to interleukin 2. One nonlimiting example of such an embodiment is a G129R variant of human prolactin linked to interleukin 2.

The PRL variants of the invention may be prepared by chemical synthesis or by recombinant DNA techniques. Generally, a cDNA of PRL may be prepared using standard PCR amplification techniques, RNA or cDNA prepared from a cell which produces PRL (such as a pituitary cell) as a template, and oligonucleotide

primers designed based on known PRL nucleic acid or amino acid sequence. A non-limiting example of the preparation of a cDNA encoding hPRL is set forth in Section 7, below. Alterations may then be introduced into the PRL cDNA either randomly or by directed mutagenesis. An example of the use of oligonucleotide mediated site-directed mutagenesis is also set forth in Example 7, and illustrates the introduction of the G129R substitution into hPRL.

Where the PRL variant is to be produced by recombinant techniques, a nucleic acid encoding the PRL variant may be incorporated into an expression vector, operatively linked to a suitable promoter/enhancer sequence. The expression vector may further contain one or more elements which aid in the expression of the PRL variant, including a transcription termination site, a polyadenylation site, a ribosome binding site, a signal sequence, etc. Suitable expression systems include mammalian cells, insect cells, plant cells, yeast cells, slime mold, and organisms, including transgenic plants and transgenic animals. Suitable expression vectors include herpes simplex viral based vectors such as pHSV1 (Geller et al., 1990, Proc. Natl. Acad. Sci. U.S.A. 87:8950-8954); retroviral vectors such as MFG (Jaffee et al., 1993, Cancer Res. 53:2221-2226), and in particular Moloney retroviral vectors such as LN, LNSX, LNCX, LXSN (Miller and Rosman, 1989, Biotechniques 7:980-989); vaccinia viral vectors such as MVA (Sutter and Moss, 1992, Proc. Natl. Acad. Sci. U.S.A. 89:10847-10851); adenovirus vectors such as pJM17 (Ali et al., 1994, Gene Therapy 1:367-384; Berker, 1988, Biotechniques 6:616-624; Wand and Finer, 1996, Nature Medicine 2:714-716); adeno-associated virus vectors such as AAV/neo (Mura-Cacho et al., 1992, J. Immunother. 11:231-237); lentivirus vectors (Zufferey et al., 1997, Nature Biotechnology 15:871-875); plasmid vectors such as pCDNA3 and pCDNA1 (InVitrogen), pET 11a, pET3a, pET11d, pET3d, pET22d, and pET12a (Novagen); plasmid AH5 (which contains the SV40 origin and the adenovirus major late promoter), pRC/CMV (InVitrogen), pCMU II (Paabo et al., 1986, EMBO J. 5:1921-1927), pZipNeo SV (Cepko et al., 1984, Cell 37:1053-1062), pSRα (DNAX, Palo Alto, CA) and pBK-CMV; and baculovirus expression vectors (O'Reilly et al., 1995, Baculovirus Expression Vectors, Oxford University Press), such as p2Bac (InVitrogen).

A PRL variant produced in a recombinant expression system may then be purified by standard techniques, including electrophoresis, chromatography (including affinity chromatography), and ultrafiltration.

5.2. TRUNCATED PROLACTIN RECEPTORS

The present invention provides for cell-free truncated prolactin receptors (referred to herein as PRL-BP(s)), which retain the ability to bind to PRL and therefore are able to compete with the cell surface forms of PRLR for PRL binding, thereby inhibiting the ability of PRL to interact with its receptor.

A PRL-BP may be prepared by removing all or a part of the transmembrane and/or intracellular domains of the PRLR, either enzymatically or using recombinant DNA techniques. In a specific, nonlimiting embodiment of the invention, the PRLR to be truncated is as described in Boutin et al., 1989, Mol. Endocrinol. 3:1455-1461.

For recombinant preparation, nucleic acid molecules encoding the native prolactin receptor may be prepared and then altered to encode a PRL-BP. For example, but not by way of limitation, the PRLR may be cloned using techniques as set forth in Example 9, below.

The amino acid sequence of PRLR from a variety of different organisms is known. The human PRLR sequence is obtainable from Genbank Accession No: 13032. Further, the amino acid residues which delineate the extracellular, transmembrane and cytoplasmic domains of the PRLR are also known (see for example, Kelly et al., 1989, Biol Reprod 40:27-32). Given the elucidation of these domains, one skilled in the art would readily be capable of producing a truncated form of PRLR which retains the ability to bind PRL, but which may by used to inhibit the effects of PRL.

Recombinant DNA methods which are well known to those skilled in the art can be used to construct expression vectors containing PRL-BP coding sequences and appropriate transcriptional/translational control signals. The efficiency of expression can be enhanced by the inclusion of appropriate transcriptional enhancer elements, transcriptional terminators, etc.. The methods may include *in vitro*

recombinant DNA and synthetic techniques and *in vivo* recombinants (See, for example, Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Press, Cold Spring Harbor, N.Y., and Glover, D.M., (ed.), 1985, DNA Cloning : A Practical Approach MRL Press, LTD., Oxford, U.K., Vol.I,II) which are incorporated by reference herein in their entirety.

When recombinant DNA technology is used to produce PRL-BP, it may be advantageous to engineer fusion proteins that can facilitate, for example, solubility or purification. Such fusion proteins can be made by ligating the appropriate nucleic acid sequences encoding the desired amino acid sequences to each other by methods known in the art, in the proper reading frame, and expressing the fusion protein by methods commonly known in the art. The PRL-BP gene product contained within such fusion proteins can comprise, for example, one or more of the extracellular domains or portions, preferably the ligand-binding portion.

In one specific example, for hPRL-BP expression, a mammalian expression vector such as pcDNA3.1/His Xpress (Invitrogen Corp., San Diego, CA) may be used. This vector contains a human immediate-early cytomegalovirus promoter and bGH poly A addition signal. In addition, it offers an in frame (His)6 peptide at the N-terminus which allows an easy detection after purification of hPRL-BP. Recombinant hPRL-BP produced using such a vector in cell culture may be concentrated by ultrafiltration, using techniques as set forth in Section 7 below. The concentration of hPRL-BP following ultrafiltration may be determined by protein assay and confirmed by Western Blot analysis using anti-His antibody (Santa Cruse, CA) and may be quantified by densitometric methods(Fernadez and Kopchick, 1990, Anal. Biochem. 191:268-271).

Alternatively, a truncated PRL-BP may be made by protein synthesis techniques, *e.g.*, by use of a peptide synthesizer. In addition, truncated PRL-BP may be prepared by purification of full length PRLR protein, from either naturally occurring or genetically engineered PRLR producing cells, followed by enzymatic cleavage of the purified protein using proteolytic enzymes, such as trypsin, to form PRL-BP.

5.3. SCREENING ASSAYS FOR IDENTIFICATION OF PRLR AGONISTS AND ANTAGONISTS

The present invention provides a cell-based assay system that can be used to identify compounds or compositions that modulate PRLR activity, and therefore, may be useful for regulation of cell proliferation and treatment of diseases associated with aberrant cell proliferation. The cell-based assay system of the invention is designed to assay for cellular apoptosis. The assay system is based on the observation that the PRLR antagonist G129R is capable of inducing apoptosis in cells expressing the PRLR.

In accordance with the present invention, a cell-based assay system is provided to screen for compounds that modulate the activity of PRLR, and thereby, modulate cell proliferation. Compounds that may affect PRLR activity include but are not limited to compounds that bind to the PRLR and either activate signal transduction (agonists) or block activation (antagonists). The invention assay systems provide rapid, reliable methods to identify compounds which interact with, and thereby affect the function of PRLR.

A method for identifying a compound capable of modulating prolactin receptor activity, comprises the following steps:

a. contacting a compound to a cell that expresses the prolactin receptor;

b. measuring the level of apoptosis in the cell; and

c. comparing the level of apoptosis obtained in (b) to the level obtained in the absence of the compound;

such that if the level obtained in (b) differs from that obtained in the absence of a compound, a compound capable of modulating prolactin receptor activity has been identified. If the level of apoptosis is increased in such an assay an antagonist of the prolactin receptor has been identified.

In yet another embodiment of the invention, a method for identifying a compound capable of inducing the activity of the prolactin receptor, is provided that comprises the following steps:

a. contacting a compound to a cell that expresses the prolactin receptor, in the presence and absence of a compound that induces prolactin receptor mediated apoptosis;

b. measuring the level of apoptosis in the cell in the presence and absence of the compound that induces prolactin receptor mediated apoptosis; and

c. comparing the levels of apoptosis obtained in (b);

such that if the level of apoptosis is decreased in the presence of the compound that induces prolactin receptor mediated apoptosis, a compound capable of activating the activity of the prolactin receptor has been identified.

To this end, cells that endogenously express PRLR can be used to screen for compounds that modulate the activity of the receptor. In a preferred embodiment of the invention the cells are transformed cells, such as for example, breast cancer cells or prostate cancer cells. In addition, cells that do not normally express PRLR can be genetically engineered to express the PRLR gene and such cells may be used for screening purposes. Those of skill in the art recognize that any cell line capable of transfection, and having low to no background level of the PRLR is acceptable.

In utilizing such cell-based assay systems, the cells expressing PRLR are exposed to a test compound or to vehicle controls (e.g., placebos). In assays designed for identification of PRLR agonists, compounds that induce PRLR mediated apoptosis, such as G129R, are also added to the assay. After exposure, the cells can be assayed to measure for the level of apoptosis. Assays designed to measure apoptosis include the terminal deoxynucleotidly transferase mediated dUTP nick end labeling (TUNEL) assay (Kebers et al., 1998, Experimental Cell Research 240:197-205); assays to detect activated caspases (Janicke et al., 1998, J. Biol. Chem. 273:9357-9360); DNA ladder gel assays to detect fragmented DNA by gel electrophoresis (Bursch et al., 1996, Carcinogenesis 17:1595-1607); assays to detect *bcl*-2 and *bax* protein levels (Wuerzberger et al., 1998, Cancer Research 58:1876-1885); Hoechst/DAPI staining to detect nuclear condensation in apoptotic cells (Bursch et al., 1998. Carcinogenesis 17:1595-1607); Annexin V staining of phospatidyl serine on

the cytoplasmic membrane (van Engeland et al., 1996, Cytometry 24:131-139); analysis of DNA content by propidium iodide staining followed by flow cytometry (Sherwood et al., Methods in Cell Biology 46:77-97; and morphological studies using electron and phase contrast microscopy (Bursch et al., Carcinogenesis 17:1595-1607).

The ability of a test compound to induce the level of apoptosis, above those levels seen with cells treated with a vehicle control, indicates that the test compound acts as an antagonist to inhibit signal transduction mediated by PRLR. In contrast, the ability of a test compound to reduce the level of apoptosis in the presence of compounds such as G129R, above those levels seen with cells treated with a vehicle control, indicates that the test compound induces signal transduction mediated by PRLR.

High throughput screening can be accomplished by plating the test cells into wells of microtiter plates, each of which will contain a potential PRLR antagonist or agonist. The wells will also contain complete medium, and in instances where an agonist is to be identified a compound such as G129R is included. After incubation with potential antagonists or agonists, the cells are assayed for apoptosis using methods such as those described above. Potential antagonists are those compounds that induce apoptosis in cells expressing the PRLR. Potential agonists are those compounds that compete with G129R for receptor binding and thereby inhibit G129R induced apoptosis.

The compounds which may be screened in accordance with the invention include, but are not limited to inorganic compounds, peptides, antibodies and fragments thereof, and other organic compounds (e.g., peptidomimetics) that bind to PRLR and either activate the activity of PRLR (i.e., agonists) or inhibit the activity of PRLR (i.e., antagonists). Compounds may include, but are not limited to, peptides such as, for example, soluble peptides, including but not limited to members of random peptide libraries; (see, e.g., Lam et al., 1991, Nature 354:82-84; Houghten et al., 1991, Nature 354:84-86), and combinatorial chemistry-derived molecular library made of D- and/or L- configuration amino acids, phosphopeptides (including, but not limited to, members of random or partially degenerate directed phosphopeptide libraries; see, e.g., Songyang et. al., 1993, Cell 72:767-778). Screening the libraries

can be accomplished by any of a variety of commonly known methods. In a specific embodiment of the invention, peptide variants of PRL may be screened for their ability to regulate the activity of the PRLR.

Compounds identified via assays such as those described herein may be useful, for example, for ameliorating diseases associated with aberrant cell proliferation. Assays for testing the efficacy of compounds identified in the screens can be tested in animal model systems for proliferative disorders, such as cancer.

5.4. UTILITIES OF THE INVENTION

The present invention provides for methods and compositions whereby a PRL variant (which acts as a PRL antagonist) or a truncated form of the PRLR (which competes with endogenous receptor for PRL binding) may be used to inhibit the effects of PRL, and in particular, may be used to inhibit PRL-mediated cell proliferation. The method of the invention comprises the administration of a prolactin variant, or a truncated form of the PRLR, to a subject having a proliferative condition wherein the proliferating cells express a prolactin receptor.

In particular specific nonlimiting embodiments, a PRL variant or a truncated PRLR (also referred to as a PRL-BP) of the invention may be tested for the ability to antagonize PRL activity in a panel of cell lines expressing different levels of the PRLR and/or PRL, so as to permit the inference of an effect which varies according to PRL/PRLR availability. For example, the activity of a hPRL variant or a truncated hPRLR may be tested in all or a subset of the following five different human breast cancer cell lines (T-47D, MCF-7, HTB19, HTB20, and HTB123 from ATCC). The hPRL receptor numbers on these cell lines have been reported to be: T-47D (25,800/cell), MCF-7 (8,300/cell), HTB19 (6,435/cell), HTB20 (5,480/cell), HTB123 (1,094/cell, normal breast cell=1,700/cell). Therefore, these cell lines represent a spectrum of hPRL receptor levels on human breast cancer cells. It should be noted that the use of human breast cancer cell lines is preferred over the use of the rat Nb2 T-cell lymphoma cell line, widely used in the lactogenic hormone studies, in order to avoid the potential confusing effects caused by species specificity. Assays which may be used to determine the effects of the PRL variant or the truncated PRLR include (i)

(for variant PRL) a competitive receptor binding assay, to examine if the antagonists are competing at the receptor level; (ii) detection/quantitation of phosphorylation of STAT 5 protein to examine if the putative antagonist inhibits the intracellular signal transduction induced by PRL; and (iii) a cell proliferation assay, which is used as an overall test for the potential inhibitory effects of a variant PRL or a truncated PRLR. One preferred method of testing the proliferative or anti-proliferative effects of PRL, variant PRL, or truncated PRLR is a mixed cell culture assay such as that diagramed in FIGURE 6 and explained in Section 8 below.

Conditions which may benefit from the administration of a PRL variant or a PRL-BP of the invention include both benign and malignant proliferation of cells which express a PRLR. Such conditions include but are not limited to proliferative diseases of the breast, including benign conditions such as breast adenomas and fibrocystic disease, and malignant conditions such as breast cancer, including ductal, scirrhous, medullary, colloid and lobular carcinomas (local or metastatic); and proliferative diseases of the prostate, including benign prostatic hypertrophy and prostate cancer (local or metastatic). Proliferative conditions involving cells which express a receptor homologous to the PRLR may also be treated, including conditions involving cells which express a growth hormone receptor.

As set forth in Example 11, below, prolactin variants are capable of inducing cellular apoptosis in human breast cancer cells and prostate cancer cells. Thus, the present invention provides methods for inducing apoptosis in cells expressing the prolactin receptor, as well as cells expressing a receptor homologous to the prolactin receptor, thereby inhibiting proliferation of such cells. In an embodiment of the invention, expression of the PRLR receptor can be targeted to a specific cell population targeted for apoptosis, such as a cancer cell population. Nucleic acid molecules expressing PRLR can be transferred into the targeted cell population using methods such as those employed in gene therapy protocols. Once expressed on the surface of the targeted cell population, the receptor can be activated through contact with prolactin variants to induce apoptosis of the targeted cell.

In the treatment of proliferative conditions, the PRL variant or PRL-BP may be administered either in isolation or as part of a sequential or combined

treatment regimen. As nonlimiting examples, where the condition to be treated is breast cancer, additional agents used in a combined regimen may include anti-estrogens such as tamoxifen and/or a chemotherapeutic agent. Where the condition to be treated is prostate cancer, additional agents used in a combination regimen may include an anti-androgen and/or a chemotherapeutic agent. A combined treatment regimen is based on the observation that the use of a prolactin variant, in combination with an anti-estrogen, such as 4-OH tamoxifen, exhibited a synergistic inhibitory effect.

The present invention accordingly provides for compositions comprising a PRL variant or PRL-BP, in a suitable pharmaceutical carrier, for use in the foregoing methods. Such compositions may be administered by any suitable technique, including local application, intravenous, intraarterial, intrathecal, intraperitoneal, oral, etc..

Pharmaceutical compositions suitable for use in the present invention include compositions containing a PRL variant or PRL-BP in an effective amount to achieve its intended purpose. More specifically, an effective dose refers to that amount of PRL variant or PRL-BP required to inhibit proliferation of cells expressing the PRLR thereby decreasing the symptoms associated with a proliferative condition. Determination of effective amounts is well within the capability of those skilled in the art.

The effective concentrations of the compounds of the invention may be established in cell culture systems and/or in transgenic animals. The effective dose may be determined using a variety of different assays. For example, cell proliferation assays may be conducted to quantitate the concentration of PRL variant or PRL-BP required to inhibit cell proliferation. In addition, assays may be performed to quantitate the concentration of PRL variant or PRL-BP required to induce cellular apoptosis. Inhibition of tumor cell growth can be assayed to detect PRL variant or PRL-BP mediated inhibition of tumor cell proliferation. In such instances, the effective dose of PRL variant or PRL-BP is that amount required to inhibit the proliferation of cancer cells and inhibit the growth of a tumor in a patient. In certain instances, it may be desirable to co-administer to a subject exhibiting a proliferative

condition, prolactin variants or PRL-BP in conjunction with, one or more, additional agent. Such agents include, for example, anti-estrogens, such as tamoxifen, or anti-androgens. Determination of effective amounts of these additional compounds is well within the capability of those skilled in the art.

The amount of the composition will, of course, also be dependent on the subject being treated, the proliferative disorder being treated, the severity of the disorder symptoms and the judgment of the prescribing physician. In some instances it may be necessary to adjust the treatment to a lower dose due to undesirable side effects as well as adjusting the treatment to higher levels if the clinical response is not adequate.

6. EXAMPLE: DESIGN OF A VARIANT PROLACTIN HAVING ANTAGONIST ACTIVITY

Since there is no crystal structural data presently available regarding hPRL, a computer algorithm program developed by Garnier et al., 1978, J. Mol. Biol. 120:97-120, was used to analyze and compare the secondary structures of hPRL and hGH. The results showed that the overall α-helix regions are very similar, suggesting that these hormones share a similar overall conformation. When the amino acid sequences in the third α-helix were compared between GHs and PRLs, it is clear that the Gly 129 of hPRL corresponds to Gly 120 of hGH and it is absolutely conserved among the GH/PRL family (Chen et al., 1994, J. Biol. Chem. 269:15892-15897). Therefore, a Gly to Arg substitution mutation in hPRL was prepared in order to generate a hPRL receptor specific antagonist.

7. EXAMPLE: PREPARATION OF THE G129R PROLACTIN VARIANT

7.1. CLONING OF THE HUMAN PROLACTIN GENE

Human PRL was successfully cloned using reverse transcription (RT) followed by polymerase chain reaction (PCR). Briefly, human pituitary polyA RNA (CloneTech, Ins. Palo Alto, CA) was used as template. A hPRL antisense primer was designed starting 2 bases from the stop codon (TAA) of hPRL cDNA (5' GCTTAGCAGTTGTTGTTGTG 3') and a sense primer was designed from ATG (5'

ATGAACATCAAAGGAT 3'). RT/PCR was carried out using a kit from Perkin-Elmer Cetus, Inc. (Norwalk, CT). The nucleotide sequence of the resulting hPRL was determined by the dideoxy chain-termination method using modified T7 DNA polymerase (Sequenase, United States Biochemical), and was found to be identical to that reported in GenBank except for a one base difference which results in a silent mutation at codon 21 (CTG->CTC). A schematic representation of the cloning process, including preparation of the pUCIG-Met expression vector, is summarized in FIGURE 1.

7.2. CREATION OF THE G129R PROLACTIN VARIANT

The parental plasmid which contains the hPRL cDNA and a M13 F1 origin of replication (FIGURE 1) was transformed into *E. coli* (CJ236). Single stranded plasmid DNA containing uridine was isolated from the transformed CJ236 bacteria using the helper bacteriophage, M13k07. Six pmol of oligonucleotide containing sequence directing the G129R mutation was annealed with 0.2 pmol of single stranded DNA in annealing buffer (200 mM Tris-HCl, 20 mM $MgCl_2$, 100 mM NaCl) by heating to 70°C for 5 minutes followed by slow cooling. The oligonucleotide (5'CGGCTCCTAGAGA**GG**ATG-GAGCT3'), which encodes the G129R mutation was used to prime synthesis of a complementary strand of DNA, using single stranded DNA as a template, that is catalyzed by T4 DNA polymerase. After synthesis, the double stranded DNA was used to transform *E. coli* (DH5a). Individual clones were isolated and screened for hPRL-G129R by DNA nucleotide sequencing. The G129R hPRL variant is hereafter referred to as hPRLA, the "A" referring to its antagonist activity.

7.3. EXPRESSION OF CLONED PROTEINS

The hPRL and hPRLA-encoding nucleic acids were each inserted into a mammalian cell expression vector in which transcription of the cDNAs is controlled by the mouse metallothionein enhancer/promoter sequence and bGH poly A addition signal (Chen et al.,1991, J. Biol. Chem. 266:2252-2258; Chen et al., 1991, Endocrinol. 129:1402-1408; Chen et al., 1991, Mol. Endocrinol. 5:1845-1852; Chen

et al., 1994, J. Biol. Chem. 269:15892-15897).To establish stable mouse L cell lines which produce hPRL and hPRLA, mouse L cells [thymidine kinase-negative (TK) and adenine phosphoribosyl transferase-negative (APRT)] were selected as an *in vitro* expression system. Stable cell lines which express hPRL (which will be used as positive control) and hPRLA (~5-10 mg/l/24h/million cells) were prepared.

Membrane ultrafiltration was used to partially purify as well as concentrate hPRL and hPRLA from conditioned cell culture media, using techniques as set forth in Chen et al., 1994, J. Biol. Chem. 269:15892-15897. The separation is based on the relative molecular size and the pore size of membrane. The ultrafiltration membranes were obtained from Amicon, Inc. (Northorough, MA). Two types of membranes were used, YM10 and YM100. A 200ml stirred cell with Amicon YM100 under 20 psia transmembrane pressure was first used for removal of large impurities from the culture media. The permeate (>90% of recovery of hPRL) was applied onto a second filtration protocol which uses YM10 membrane to reduce the volume of solution and thus concentrate the protein. The concentration of hPRL or hPRLA was determined using an immunoradiometric assay (IRMA) kit from Diagnostic Products Corp. (Los Angeles, CA).

8. EXAMPLE: INHIBITORY ACTIVITY OF THE G129R PROLACTIN VARIANT

8.1. MATERIALS AND METHODS

Radioreceptor binding assay. Purified hPRL was labeled with $Na^{125}I$ by the lactoperoxidase method to a specific activity of 80-105 $\mu Ci/\mu g$ as described in Harding et al., 1996. J. Biol. Chem.271:6708-6712. Briefly, 1.0 mCi of $Na^{125}I$ was added to 1 mg of hPRL. Lactoperoxidase (10 μg dissolved in 10 μl of 0.4 mol/liter acetate butter, pH 5.6) and H_2O_2 (5 μl of 1.76 mmol/liter) were then added. After 30 min, the reaction was terminated by the addition of 100 μl of transfer buffer (0.47 mol/liter sucrose, 0.06 mol/liter KI, sodium azide 0.02%, pH 7.6). Radiolabeled hPRL was then separated by Sephadex G-100 chromatography. Human breast cancer cells were plated in 6-well plates. After preincubation in serum-free DMEM for 2-3 hours to deplete serum, the monolayer of cells was exposed to serum-free conditioned medium containing ^{125}I-hPRL (50,000 cpm) in the presence of various concentration

of hPRL or hPRLA for 2-3 hours at 37°C. After incubation at room temperature for 3 hours, the cells were washed with phosphate-buffered saline (PBS) two times, and then lysed in 1 ml of 1% SDS/0.1N NaOH. The CPM in lysates were then determined. Non-specific binding was measured by adding 5 μg/ml of unlabeled hPRL in regular mouse L cell conditioned media to control nonspecific displacement.

Assay of hPRL induction of tyrosine phosphorylation of STAT5 protein. STAT proteins represent a family of proteins, having molecular masses of approximately 92-95 kDa, which have been found to be tyrosyl phosphorylated when GHR or PRLR containing cells are treated with GH or PRL, respectively. Tyrosyl phosphorylation of STAT 5 is a receptor mediated event and is thought to be an important step in ligand-induced signal transduction (Wakao et al., 1994, EMBO J. 13: 2182-2191; Kazansky et al., 1995, Mol. Endocrinol. 9:1598-1609; Waxman et al., 1995, J. Biol. Chem. 270:13262-13270). This assay was used to evaluate the ability of hPRL and hPRLA to inhibit induction of STAT 5 phosphorylation by wild type PRL.

Briefly, human breast cancer cells were plated in 12-well plates. After pre-incubation in serum-free DMEM for 2-3 hours, the cells were exposed to various concentration of hPRL and hPRLA in serum-free DMEM. The cells were incubated for 15 min at 37°C, washed once with PBS, and lysed in 300 μl lysis buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 1% β-mecaptoethanol, 0.1M DTT, 5% Sucrose, 100uM Sodium Orthovanadate, and 0.6% bromphenol blue). Thirty microliters of cell lysates were subjected to 4-12.5% SDS-PAGE and immunoblot analysis using horse radish peroxidase (HRP)-conjugated anti-phosphotyrosine antibody PY20 and ECL reagent kit (Amersham, IL). Blots were then exposed to X-ray films and developed using standard procedures (Kodak, Rochester, NY). This assay has been described in Chen et al., 1994, J. Biol. Chem. 269: 15892-15897; Chen et al., 1995, Endocrinol. 136:660-667; Wang et al., 1994, Proc. Natl. Acad. Sci. U.S.A. 91:1391-1395; Chen et al., 1995, Mol. Endocrinol. 9(3):292-302; Harding et al., 1996, J. Biol. Chem. 271(12):6708-6712.

Cell proliferation assays. hPRLA was tested for its ability to inhibit breast cancer cell proliferation in tissue culture. The human breast cancer cells were grown in corresponding culture media according to ATCC recommendations. Cells

were maintained at 37°C in a humidified atmosphere of 5% CO_2 in air. The assay conditions were essentially as described by Ginsburg and Vonderharr (1995, Cancer Res. 55:2591-2595). For individual growth experiments, cells were plated in 12 well culture plates at a density of approximately $2x10^4$ /ml, 1ml/ well. Cells were then allowed to attach for one day (T-47D, MCF-7, HTB19, and HTB20 cells, except for HTB 123, which is a suspension cells), then the overlying media was removed and changed to serum-free conditions with media containing ITS^+ (insulin-transferring-selenium-BSA-linoleic acid culture supplement; Collaborative Research Bedford, MA). Varying concentrations of hPRL alone or in combination with hPRLA were introduced. After an additional three days in culture, cells were harvested after brief trypsinization and counted in a cell counter.

For certain experiments, a mixed cell culture assay was used, diagramatically represented in FIGURE 6. In this assay, breast cancer cells were co-cultured with expressor cells which had been transfected with nucleic acid encoding PRL or a PRL variant and expressing those recombinant proteins. By varying the number of expressor cells, the amount of PRL or PRL variant present in the mixed cell culture was increased or decreased. As shown in FIGURE 6, a fixed number of breast cancer cells (T47D) were added to wells of a multi-well cell culture plate. In certain wells, which served as a control, no expressor cells were added. Then, increasing numbers of expressor cells (transfected L cells expressing either hPRL (L-PRL) or hPRLA (L-PRLA)) were added to breast cancer cell-containing wells to create mixed cultures. The same numbers of expressor cells were cultured in parallel (without T47D cells) to serve as controls. After culturing under standard conditions for a period of time, the number of cells present in the wells was counted, and the number of L cells in the corresponding control culture was subtracted. The resulting number could then be compared to the number of T47D cells in the T47D control culture to evaluate the effects of the recombinant product on breast cancer cell proliferation.

8.2. RESULTS AND DISCUSSION

Results of radioreceptor binding assay. The results of the assay performed using T-47D and HTB123 cells along with a panel of human cancer cells are shown in FIGURE 2. They demonstrate that two cell lines (T-47D and HTB123) among those tested contain minimum hGH receptor specific binding as compared to human leukemia cells, lymphoma cells and retinoblastoma cells.

Phosphorylation of STAT5 proteins. Experiments testing the abilities of hPRL and hPRLA, and combinations thereof, to induce phosphorylation of STAT5 proteins in T-47D human breast cancer cells have demonstrated that hPRLA is able to block the signal transduction induced by hPRL (FIGURE 3), thereby demonstrating the antagonistic activity of PRLA. In particular, FIGURE 3 shows that the induction of phosphorylation of STAT5 proteins induced by hPRL (lane 2) was absent in the presence of hPRLA only (lane 3), is partially eliminated when equal amounts of hPRL and hPRLA were present (lane 4), and is undetectable when there was an excess of hPRLA (lane 5).

Cell proliferation assays. Cell proliferation assay results from experiments in which T-47D cells were exposed to either hGH or hPRL are shown in FIGURE 4. The bell shaped dose response curves suggest that similar mechanisms (i.e., one ligand leading to dimerization of receptors) are used by both GH and PRL signal transduction. Since the affinity of binding site one of the ligand is apparently much higher than the affinity at binding site two, at high concentrations of hormone, all receptors are occupied by a single ligand via the high affinity site (the "self-antagonism" phenomenon). FIGURE 5A-B compares the effects of hPRL and hPRLA (the G129R variant of human prolactin) (FIGURE 5A) to the effects of estrogen and the estrogen antagonist tamoxifen (FIGURE 5B). While hPRL and estrogen increased proliferation of T47D cells (relative to untreated control cultures), hPRLA and tamoxifen had a comparable inhibitory effect.

FIGURES 7 and 8 depict the results of mixed cell culture assays in which a varying number of transfected L cells (shown on the y-axis) expressing hPRL or hPRLA (the G129R variant of human prolactin) were co-cultured with T47D human breast cancer cells for 24 or 72 hours (FIGURE 7) or for one, two, three or five

days (FIGURE 8). While hPRL resulted in an increase in T47D proliferation (relative to untreated T47D cell cultures), hPRLA inhibited proliferation by up to 100 percent.

FIGURE 9A-B compares the inhibitory effects of hPRLA in mixed cell culture on the two different human breast cancer cell lines T47D and MCF-7 (FIGURES 9A and 9B, respectively). hPRLA expressed by transfected L cells had an inhibitory effect on both cell lines, but the effect was greater on T47D cells, probably because there are a greater number of prolactin receptors on T47D cells relative to MCF-7 cells (Shiu et al., 1979, Cancer Res. 39:4381-4386); Ormandy et al., 1997, J. Clin. Endocrinol. Metab. 82:3692-3699).

9. EXAMPLE: CLONING OF THE PROLACTIN RECEPTOR

hPRL-BP cDNA was cloned using reverse transcription (RT) followed by the polymerase chain reaction (PCR). The hPRL-BP antisense primer was designed at a NcoI restriction enzyme cutting site which is located 66 bases from the putative transmembrane domain and a stop codon (TGA) was incorporated (5' GCACT**TCA**GTATCCATGGTCTGGT 3'). The sense primer was designed including translational start codon ATG (5' AGAAGGCAGCCAAC**ATG**AAG 3'). RT/PCR was carried out by using a kit from Perkin-Elmer Cetus, Inc. (Norwalk, CT). The nucleotide sequence hPRL-BP was determined by the dideoxy chain-termination method using modified T7 DNA polymerase (Sequenase, United States Biochemical).

10. EXAMPLE: INHIBITORY EFFECTS OF A PROLACTIN ANTAGONIST AND ITS SYNERGISTIC ACTION IN CONJUNCTION WITH TAMOXIFEN

The subsection below describes data derived from cell proliferation assays demonstrating that a prolactin variant, when added together with an anti-estrogen agent, induces a synergistic inhibitory effect on cell proliferation.

10.1. MATERIALS AND METHODS

RT-PCR. The RT-PCR technique was used to clone hPRL cDNA. Human pituitary mRNA was purchased from Clontech Laboratory, Inc. (Palo Alto, CA 94303). A RT-PCR kit was from Perkin-Elmer, Inc. (Norwalk, CT). The hPRL antisense primer (for the RT reaction) was designed 2 bases from the stop codon (in bold) of hPRL cDNA (5' GC**TT**AGCAGTTGTTGTTGTG 3') and the sense primer was designed from the translational start codon ATG (5' **ATG**AACATCAAAGGAT 3'). The RT-PCR reaction was carried out following the manufacturer's recommendation. The PCR product was then cloned into an expression vector pcDNA3.1 from Invitrogen Corp. (Carlsbad, CA). The expression of hPRL cDNA was controlled by the human immediate-early cytomegalovirus (CMV) enhancer/promotor and a polyadenylation signal and transcription termination sequence from the bovine GH gene. This vector also contains a neomycin gene that allows for selection of neomycin resistant mammalian cells (FIGURE 1B).

Rational Design of Hprl-G129R. The amino acid sequences of all known PRLs in the third α-helical region and aligned them with GH sequences. It is clear that Gly 129 of hPRL is invariable among PRLs and corresponds to hGH 120 suggesting a potentially important role in its function. We, therefore, decided to make a single amino acid substitution mutation at Gly 129 of hPRL (hPRL-G129R). We have used a similar approach to that which we have successfully previously used in the discovery of hGH antagonists, in hope of producing a hPRLR specific antagonist (FIGURE 11).

Oligonucleotide Directed Mutagenesis hPRL-G129R cDNA was generated using PCR mutagenesis protocol. Oligonucleotides containing the desired mutation (5' CTTCTAGA**GC**GCATGGAGCTCATA 3'; and (5' CCCTCTAGACTCGAGCGGCCGCC3') were synthesized by National Biosciences, Inc. (Plymouth, MN). The codon for 129 Arg is in bold and the restriction site XbaI is underlined. The PCR product was digested with XbaI and ligated back into the previously described vector (FIGURE 1B). The mutation was then confirmed by DNA nucleotide sequencing.

Cell Lines. Two human breast cancer cell lines (T47-D and MCF-7) and a mouse L fibroblast cell line were acquired from ATCC. Both human breast cancer cell lines have been characterized as estrogen receptor (ER) positive and PRLR positive cell lines (Ormandy, C.J., et al., 1997, J. Clin. Endocrinol. Metabo 82:3692-99). Cells were grown routinely as a monolayer culture in Dulbecco's Modified Eagle's medium (DMEM) for MCF-7 and L cells and RPMI-1640 medium was used for T47-D after supplement with 10% fetal calf serum that was treated with dextran-coated charcoal (DCC-FCS). Media for human breast cancer cells were used without phenol red (to avoid its potential estrogen-like activities). Cell cultures were maintained at 37°C in a humidified atmosphere with 5%CO_2 and passed twice a week.

Expression and Production of hPRL and hPRL-G129R Proteins

Mouse L cell transfection and stable cell selection were performed as previously described with minor modifications (Zhou, Y. et al., 1996, Gene 177:257-129; Sun, X.Z. et al., 1997, J. Steroid Biochem. Mol. Biol. 63:29-36). Briefly, cells were plated in a 6-well plate and cultured until the culture was 50% confluent. On the day of transfection, cells were washed once with serum free media and cultured in 1 ml serum free media containing 1 μg of pcDNA3-hPRL or pcDNA3-hPRL-G129R and 10 μl LipofectAmine (GibcoBRL) for 5 h. Two milliliters of growth medium were added to the DNA/lipofectAmine solution and incubation continued. After 18-24 hours of incubation, fresh growth medium was used to replace the medium containing DNA/lipofectAmine mixture. At 72 hours after transfection, cells were diluted 1:10 and passed into the selective medium (400 μg/ml G418) to select for *neo* gene expression. Individual colonies were isolated and expanded. The expression levels of the individual cell lines were determined by using an immunoradiometric assay (IRMA) kit from Diagnostic Products Corp. (Los Angeles, CA). The cell lines with high expression levels were expanded.

Conditioned media containing hPRL and hPRL-G129R was prepared as follows. Stable cells were plated in T-150 culture flasks at 85 to 90% confluency. The growth medium were then replaced with 50 ml of RPMI-1640 containing 1% DCC-FCS and collected every other day for three times. The collected media were

then pooled and filtered through a 0.2 μm filter unit to remove cell debris and stored at –20°C until use. The concentration of hPRL or hPRL-G129R was determined by hPRL IRMA. Each batch product was further verified using a Western blot analysis protocol (Fernandex, E. et al., 1990, Anal. Biochem. 191:268-271). We have used this protocol in hGH analog studies including hGH antagonist for *in vitro* studies (Chen, W.Y. et al., 1994, J. Biol. Chem. 269:15892-15897).

Tyrosine Phosphorylation of STAT Proteins in T47-D cells. This assay is designed to examine the effects of hPRL and hPRL-G129R on signal transduction using T47-D cells as model target cells. Briefly, T47-D cells were plated in 12-well plates. After pre-incubation in serum-free medium for 2-3 hours, cells were exposed to various concentrations of hPRL or hPRL-G129R or a combination of hPRL and hPRL-G129R in serum-free medium. The cells were incubated for 15 min at 37°C, washed once with PBS, and lysed in 200 μl lysis buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 1% ß-mecaptoethanol, 0.1M DTT, 5% sucrose, 100 μM sodium orthovanadate, and 0.6% bromophenol blue). Thirty microliters of cell lysate are then subjected to 4-12.5% SDS-PAGE using the Bio-Rad Protein II system. After electrophoresis, the gels were transferred to a Hybond-ECL membrane (Amersham, IL) at 100 volts constant voltage for 2 hrs. Blots were incubated in a blocking solution of 4% BSA (Boehringer Mannheim, IN) in rinsing buffer (10 mM Tris-HCl pH 7.5, 75mM NaCl, 0.1% Tween 20, 1 mM EDTA) for 2 hrs and subsequently washed twice with rinsing buffer for 15 min. Blots were incubated with horseradish peroxidase (HRP)-conjugated anti-phosphotyrosine antibody PY20 (Amersham, IL) at a concentration of 0.1 μg/ml in the blocking solution for 1 hr. After incubation, blots were washed with rinsing buffer (15 min. each for 2 times) and developed with an ECL reagent kit according to manufacturer's suggestions (Amersham, IL). Blots were then exposed to X-ray film and developed using standard procedures (Kodak, Rochester, NY).

HPRLG129R Conditioned Media. The assay conditions were modified from that described by Ginsburg and Vonderharr (1995, Cancer Res. 55:2591-2595). T47-D cells were trypsinized and passed into 96 well plates in RPMI-1640 media containing 1% DCC-FCS in a volume of 100 μl/well. The optimal cell

number/well for each cell line was pre-determined after titration assay. For T47-D cells, 15,000 cells/well were plated. The cells were allowed to settle and adhere overnight (12-18 hours) and subsequently various concentrations of either hPRL, hRPL-G129R, E2 or 4-OH-Tamoxifen in a total volume of 100µl of culture media were added. Purified hPRL (kindly provided by Dr. Parlow, National Hormone & Pituitary Program, NIH) was used as a positive control for hPRL produced from stable L cells. Cells were incubated for an additional 96 hours at 37°C in a humidified 5% CO_2 incubator. After incubation, MTS-PMS solution (Cell Titer 96 Aqueous kit, Promega Corp.) was added to each well, following the manufacturer's instructions. Plates were read at 490 nm using a BIO-RAD benchmark microplate reader. The experiments were carried out in triplicates and repeated three to six times for each cell line.

10.2. RESULTS

Cloning and Mutagenesis of hPRL hPRL cDNA was cloned from human pituitary mRNA using RT-PCR technique. The size of the corresponding PCR product was 663 base pairs in length and it was cloned into the pcDNA 3.1 expression vector. The nucleotide sequence of hPRL was determined by the dideoxy chain-termination method using an automatic sequencer (PE Applied Biosystems, Foster City, CA). The hPRL cDNA sequence was found to be identical to that reported in GenBank except for one base difference that results in a silent mutation at codon 21 (CTG->CTC). HPRL-G129R cDNA was also generated by PCR and sequenced.

Expression of hPRL and hPRL-G129R Mouse L cell were stably transfected with either hPRL or hPRL-G129R cDNAs and neo-resistant clones were selected and expanded. Conditioned media were collected and tested for expression by use of an RIMA kit. hPRL and hPRL-G129R stable mouse L cell lines were generated that produced hPRL and hPRL-G129R in a quantity of approximately ~1 mg/L/24h/million cells (FIGURE 12).

Inhibition of Tyrosine Phosphorylation of the STAT Protein by hPRL-G129R STAT proteins represent a family of proteins with a molecular mass of approximately 92-95 kDa. The inhibitory effects of GH antagonist can be assayed by

measuring the levels of inhibition of tyrosine phosphorylation of STAT protein (Chen et al., 1994, J. Biol. Chem. 269:15892; Wang et al., Proc. Natl. Acad. Sci. USA 91:1391-1395; Silva 1993, Endocrinology 133:2307-2312). Using such an assay, the GH antagonist hGH-G120R, was demonstrated to inhibit GH induction of STAT protein phosphorylation in a dose dependent manner.

The results using hPRL and hPRL-G129R on T47-D human breast cancer cells have demonstrated that hPRL-G129R was not active in stimulating STAT protein phosphorylation. However, when hPRL-G129R was added together with hPRL, it was able to block the signal transduction induced by hPRL in a dose dependent manner (FIGURE 13) suggesting that it is functioning as a hPRL antagonist. At a 5:1 ratio, hPRL-G129R completely inhibited STAT protein phosphorylation induced by hPRL.

Human Breast Cancer Cell Proliferation Assays. Human PRL and hPRL-G129R were tested further for their ability to stimulate/inhibit breast cancer cell proliferation in cell culture. Light microscopic examination of breast cancer cell proliferation after hPRL, hPRL-G129R, E2 and 4-OH-Tamoxifen is shown in FIGURE 14A-E. It is clear that there is a significant difference in cell density between hPRL (15B), hPRL-G129R(15C) and E2 (15D), 4-OH-Tamoxifen (15E) treated cells. It is also noteworthy to point out that the overall cell condition of hPRL-G129R treated cells was not as healthy under light microscopy examination.

96 well cell proliferation assay results are shown in FIGURES 15-18. hPRL stimulated T47-D proliferation in a dose dependent manner. The maximum stimulation of hPRL (250ng/ml) was approximately 20% over basal levels after a single dose/ four-day incubation. However, when hPRL and E2 were applied simultaneously, a synergistic effect was observed. The maximum response of hPRL (100ng/ml) in the presence of 10nM of E2 was more than tripled as compared to hPRL alone (FIGURE 15).

hPRL-G129R, on the other hand, exhibited dose dependent inhibitory effects on cell proliferation (FIGURE 16A). It is noteworthy to point out that the inhibitory effect of hPRL-G129R (150ng/ml) was more potent than the maximal 500nM dose of 4-OH-Tamoxifen in the assay system (FIGURE 16B). The maximum

inhibition of a single dose of 4-OH-Tamoxifen (500nM) is approximately 15% of control (FIGURE 16B) whereas the maximum inhibition by a single dose of hPRL-G129R resulted in 25% of control (FIGURE 16A). hPRL-G129R was also able to competitively inhibit hPRL induced cell proliferation. At a 1:1 molar ratio, hPRL-G129R was able to stop the stimulatory effect of hPRL and at 2:1 molar ratio, it inhibited cell proliferation (FIGURE 17). More importantly, when hPRL-G129R was applied together with 4-OH-Tamoxifen, the inhibitory effects were doubled as compared to either the maximum dose of hPRL-G129R or 4-OH-Tamoxifen (FIGURE 18). For example, 100nM of 4-OH-Tamoxifen resulted in a 15% inhibition, yet. in the presence of 100ng/ml of hPRL-G129R the inhibitory effect resulted in approximately 32% of control.

Co-culture Experiments Stable mouse L cell lines grow at a similar rate as do regular L cells regardless of whether they are producing either hPRL or hPRL-G129R due to the fact that mouse L cells possess non-detectable PRLR (Chen, 1994, J. Biol. Chem. 269:15892-15897). The co-culture experimental set-up provides a sustained presence of biologically active hPRL-G129R, thereby resulting in a maximal response in these tumor cells.

Both human cancer cell lines after co-culture with L-G129R cells demonstrated dose dependent growth inhibition (FIGURE 19A-B). The responses were rather dramatic as compared to conditioned media experiments. Complete inhibition of cell proliferation was achieved in both cell lines. It is noteworthy that the response pattern of MCF-7 cells was shifted to the right as compared to that of T47-D cells, *i.e.* it required more hPRL-G129R to elicit the same inhibitory effects. These results can be explained by the fact that the total hPRLR number on MCF-7 cells is much less than that found on T47-D cells (Ormandy et al., Genes Dev. 15:167-178; Shih, 1981, *In*: Hormones and Breast Cancer, Cold Spring harbor Laboratory, Pike, Siiteri, and Walsh (eds) pp 185-194).

11. EXAMPLE: HUMAN PROLACTIN RECEPTOR ANTAGONIST G129R INDUCES APOPTOSIS IN MULTIPLE HUMAN BREAST CANCER CELL LINES AND PROSTATE CANCER CELLS

11.1. MATERIALS AND METHODS

Cell Lines. The human breast cancer cell lines MDA-MB-134, T-47D, BT-474 and MCF-7 were obtained from ATCC. These breast cancer cell lines were chosen based on their PRLR levels. The cell line MDA-MB-134 has the highest PRLR level followed by T-47D, BT-474, MCF-7 in decreasing order of PRLR levels (Ormundy, J Clinical Endocrinology and Metabolism 82:3692-3699).

Cell Culture. T-47D cells obtained from ATCC were grown in RPMI 1640 (phenol red free), supplemented with 10% FBS (GIBCO BRL). BT-474 cells were grown in RPMI 1640 medium (phenol red free) supplemented with 10% FBS and ATCC recommended supplements. MCF-7 cells were grown in DMEM medium (phenol red free), supplemented with 10% FBS. The cells were grown at 37°C in a humid atmosphere in the presence of 5% CO_2. The MDA-MB-134 cells were grown in Leibovitz's L-15 medium supplemented with 20% FBS and grown in CO_2 free atmosphere. The breast cancer cells were trypsinized (0.02% Trypsin – EDTA) and grown in their respective media (phenol red free) supplemented with 10% CSS (Charcoal stripped serum) for a week. Subsequently the cells were trypsinized again and plated onto an 8 chambered slide system (Lab Tek II) at a confluence of 60-70% per chamber. The next day treatments were performed on the breast cancer cells using their respective media(phenol red free), supplemented with 1% CSS. The MDA-MB-134 VI cells were grown in phenol red containing medium, but with the same serum conditions as the other breast cancer cells.

Terminal deoxynucleotidly transferase mediated dUTP nick end labeling (TUNEL) assay. Nicks of the fragmented DNA are labeled at their 3-OH ends. The fluorescein-labeled dUTP is incorporated at the 3-OH ends by using the enzyme terminal deoxynucleotidyl transferase. After the assigned period of treatment the chambers were dismantled as per the manufacturer's instructions and the TUNEL assay (Apoptosis detection system, Fluorescein- Promega) was performed as per the

manufacturer's instructions. The slide was examined under a FITC filter using an Olympus IX 70 microscope system.

11.2. RESULTS

Apoptosis (programmed cell death) is one of the central physiological mechanisms that regulates the timely and orderly death of cells (Stellar, H., 1995, Science 267:1445). The biochemical hallmark of apoptosis is internucleosomal DNA cleavage (Wyllu, 1980, Nature 284:555; Roy et al., 1992, Exp. Cell Res. 200:416-424; Wyllu, 1980, Int. Rev. Cytol. 68:251-306) and it can be detected by the TUNEL assay or by conventional gel electrophoresis (Chen, 1996, J. Cell. Biochem. 61:9-17). Cancer is a disease in which the malignant cells have a decreased ability to undergo apoptosis in response to at least some physiological stimuli (Hoffman et al., 1994, Oncogene 9:1807). Drugs that can induce cancer cells to undergo apoptosis could prove to be effective in cancer therapy.

As demonstrated herein, the PRLR antagonist G129R is able to induce apoptosis as detected by DNA fragmentation in multiple human breast cancer cell lines. FIGURE 20A-F shows that G129R induced apoptosis in a dose dependent manner after 24 h treatment and that apoptosis occurs even at physiological concentrations (50 ng/ml, FIGURE 20C). In order to demonstrate the specificity of G129R to the PRLR. hPRL (kindly provided by Dr. Parlow from NIH) and G129R were simultaneously used to treat the cells at a 1:1 and 1:4 ratio (FIGURE 20G-H). It is clear that G129R was able to compete with hPRL at a ratio of 1:1 (FIGURE 20E) and is able to competitively reverse the DNA fragmentation induced by G129R at a 4:1 ratio (FIGURE 20F). The mitogen rescue effect of hPRL is yet another indication that G129 R induces apoptosis.. The same results were obtained using BT-474 cells.

DNA fragmentation in breast cancer cells is apparent even after 2 hours of treatment by G129R at a concentration of 50 ng/ml (FIGURE 21A-D). In previous studies it was shown that 4-OH-Tamoxifen synergistically inhibited the proliferation of breast cancer cells along with G129R. Therefore, 4-OH-Tamoxifen was included in this study to verify that 4-OH-Tamoxifen also induced apoptosis in breast cancer cells by DNA fragmentation. Surprisingly, 4-OH-Tamoxifen did not induce

apoptosis in T-47D, MCF-7 or BT-474 cells at a concentration as high as 1 μM as assayed by the same protocol despite the fact that 4-OH-Tamoxifen was able to inhibit cell proliferation (FIGURE 22A-H). In contrast to 4-OH-Tamoxifen, 250 ng of G129R induced apoptosis DNA fragmentation in all four PRLR positive breast cancer cell lines after 24 hours treatment (FIGURE 23A-F).

In addition, the effect of hPRL-G129R on Caspase-3 activation was assayed in T-47D cells using an ApopAlert CPP32/ Caspase-3 assay kit (Clontech, Palo Alto, CA) as presented in FIGURE 24. T-47D cells were treated with 250 ng/ml of hPRL-G129R for 2h. The assay was performed in the presence of DEVD-CHO (caspase-3 inhibitor) to demonstrate that the Caspase-3 induction by hPRL-G129R is a specific event.

The data described above indicates that breast cancer cells are adapted to utilize prolactin as a major growth factor and undergo apoptosis when deprived of it by the competitive binding of G129R to the PRLR leading to blockage of the PRL growth signal. Thus, the continued mitogenic signal provided by hPRL may override existing apoptotic signals within breast cancer cells permitting the delayed apoptosis process to proceed. The data presented herein, indicates that the prolactin receptor antagonist G129R can be used in endocrine therapy in conjunction with tamoxifen, or by itself, in the treatment of breast cancer.

In addition, two prostate cancer cells, underwent apoptosis in response to treatment with 250ng hPRL-G129R for 24 hours as detected using TUNEL assay (FIGURE 25). The samples were in duplicate and each sample constituted about 2 million cells

The present invention is not to be limited in scope by the specific embodiments described herein which are intended as single illustrations of individual aspects of the invention, and functionally equivalent methods and components are within the scope of the invention. Indeed, various modifications of the invention, in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the claims. Various publications

are cited herein, the contents of which are hereby incorporated, by reference, in their entireties.

CLAIMS

1. A method of inhibiting the proliferation of a breast cancer cell which expresses a prolactin receptor, comprising exposing the cell to an effective concentration of a variant of human prolactin having a substitution of the glycine at position 129.

2. The method of claim 1, wherein the variant of human prolactin has a substitution of the glycine at position 129 with arginine.

3. The method of claim 1, which is used in the treatment of breast cancer in a subject in need of such treatment.

4. The method of claim 3, wherein the variant of human prolactin is administered to the subject as part of a combined therapy regimen.

5. The method of claim 4, wherein the combined therapy regimen comprises administration of an anti-estrogen agent.

6. The method of claim 5, wherein the anti-estrogen agent is tamoxifen.

7. A method of inhibiting the proliferation of a prostate cancer cell which expresses a prolactin receptor, comprising exposing the cell to an effective concentration of a variant of human prolactin having a substitution of the glycine at position 129.

8. The method of claim 7, wherein the variant of human prolactin has a substitution of the glycine at position 129 with arginine.

9. The method of claim 7, which is used in the treatment of prostate cancer in a subject in need of such treatment.

10. The method of claim 9, wherein the variant of human prolactin is administered to the subject as part of a combined therapy regimen.

11. The method of claim 10, wherein the combined therapy regimen comprises the administration of an anti-androgen.

12. A method of inhibiting the proliferation of a breast cancer cell which expresses a prolactin receptor, comprising exposing the cell to an effective concentration of a cell-free truncated prolactin receptor.

13. The method of claim 12, which is used in the treatment of breast cancer in a subject in need of such treatment.

14. The method of claim 13, wherein the variant of human prolactin is administered to the subject as part of a combined therapy regimen.

15. The method of claim 14, wherein the combined therapy regimen comprises administration of an anti-estrogen agent.

16. The method of claim 15, wherein the anti-estrogen agent is tamoxifen.

17. A method of inhibiting the proliferation of a prostate cancer cell which expresses a prolactin receptor, comprising exposing the cell to an effective concentration of a cell-free truncated prolactin receptor.

18. The method of claim 17, which is used in the treatment of prostate cancer in a subject in need of such treatment.

19. The method of claim 18, wherein the variant of human prolactin is administered to the subject as part of a combined therapy regimen.

20. The method of claim 19, wherein the combined therapy regimen comprises the administration of an anti-androgen.

21. A method for inducing cellular apoptosis in a cell expressing the prolactin receptor comprising exposing the cell to an effective concentration of a variant of human prolactin having a substitution of the glycine at position 129.

22. The method of claim 21, wherein the variant of human prolactin has a substitution of the glycine at position 129 with arginine.

23. The method of claim 21 wherein the cell is genetically engineered to express the prolactin receptor.

24. A method for identifying a compound capable of modulating prolactin receptor activity, comprising:

d. contacting a compound to a cell that expresses the prolactin receptor;

e. measuring the level of apoptosis in the cell; and

f. comparing the level of apoptosis obtained in (b) to the level obtained in the absence of the compound;

such that if the level obtained in (b) differs from that obtained in the absence of a compound, a compound capable of modulating prolactin receptor activity has been identified.

25. The method of claim 24 wherein the compound increases the level of apoptosis in the cell.

26. The method of claim 24 wherein the compound decreases the level of apoptosis in cell in the presence of a prolactin receptor antagonist.

FIGURE 1A

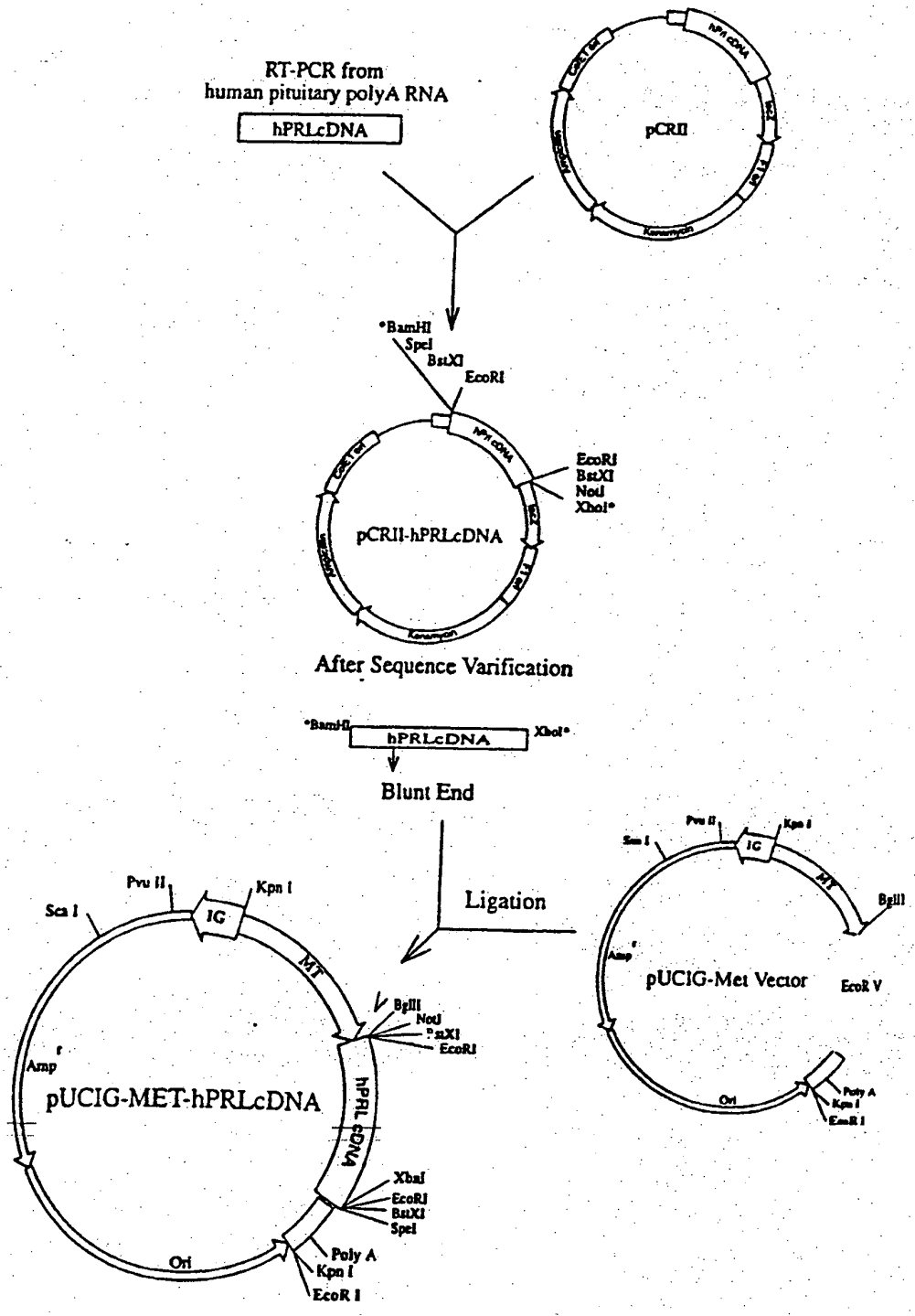

RT-PCR from
human pituitary polyA RNA
hPRLcDNA
pCRII
*BamHI
SpeI
BstXI
EcoRI
pCRII-hPRLcDNA
EcoRI
BstXI
NotI
XhoI*
After Sequence Varification
*BamHI
hPRLcDNA
XhoI*
Blunt End
Ligation
Pvu II
Kpn I
Sca I
IG
MT
BglII
NotI
BstXI
EcoRI
Amp
pUCIG-MET-hPRLcDNA
hPRL cDNA
XbaI
EcoRI
BstXI
SpeI
Ori
Poly A
Kpn I
EcoR I
pUCIG-Met Vector
EcoR V

FIGURE 1B

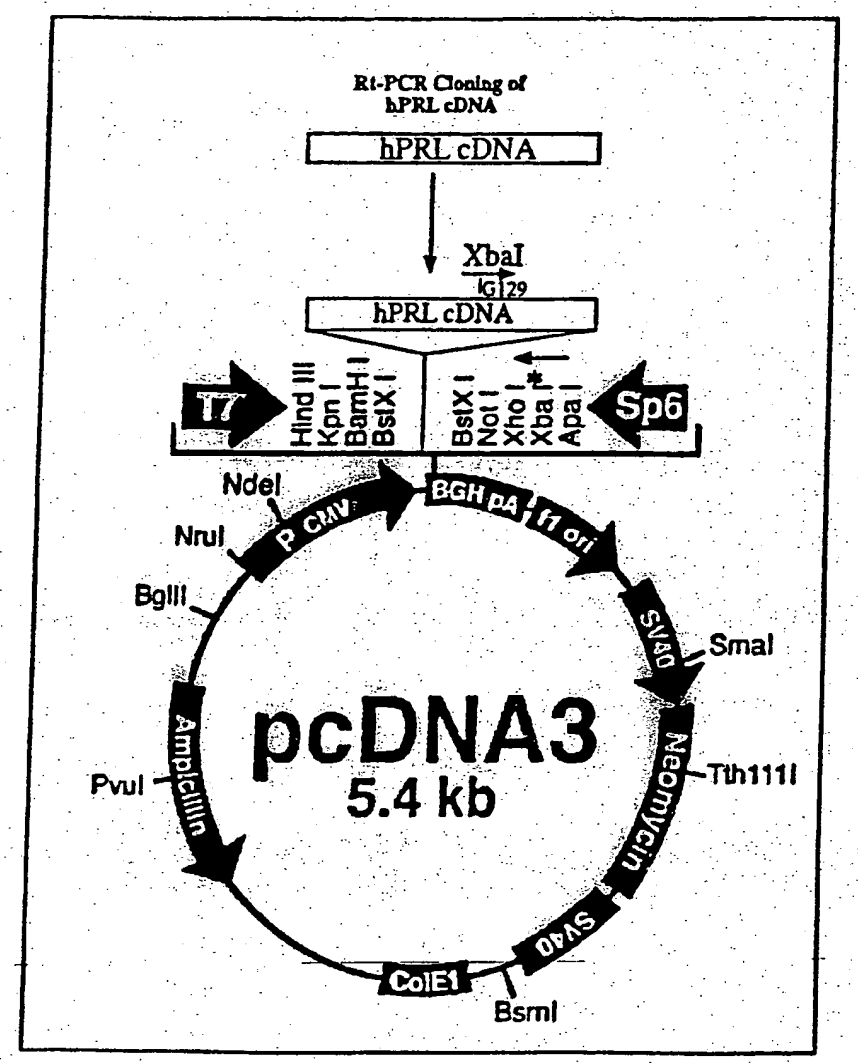

Rt-PCR Cloning of hPRL cDNA
hPRL cDNA
XbaI
hPRL cDNA
T7
Hind III
Kpn I
BamH I
BstX I
BstX I
Not I
Xho I
Xba I
Apa I
Sp6
NdeI
NruI
BglII
P CMV
BGH pA
f1 ori
SV40
SmaI
pcDNA3
5.4 kb
Neomycin
Tth111I
Ampicillin
PvuI
SV40
ColE1
BsmI

FIGURE 2

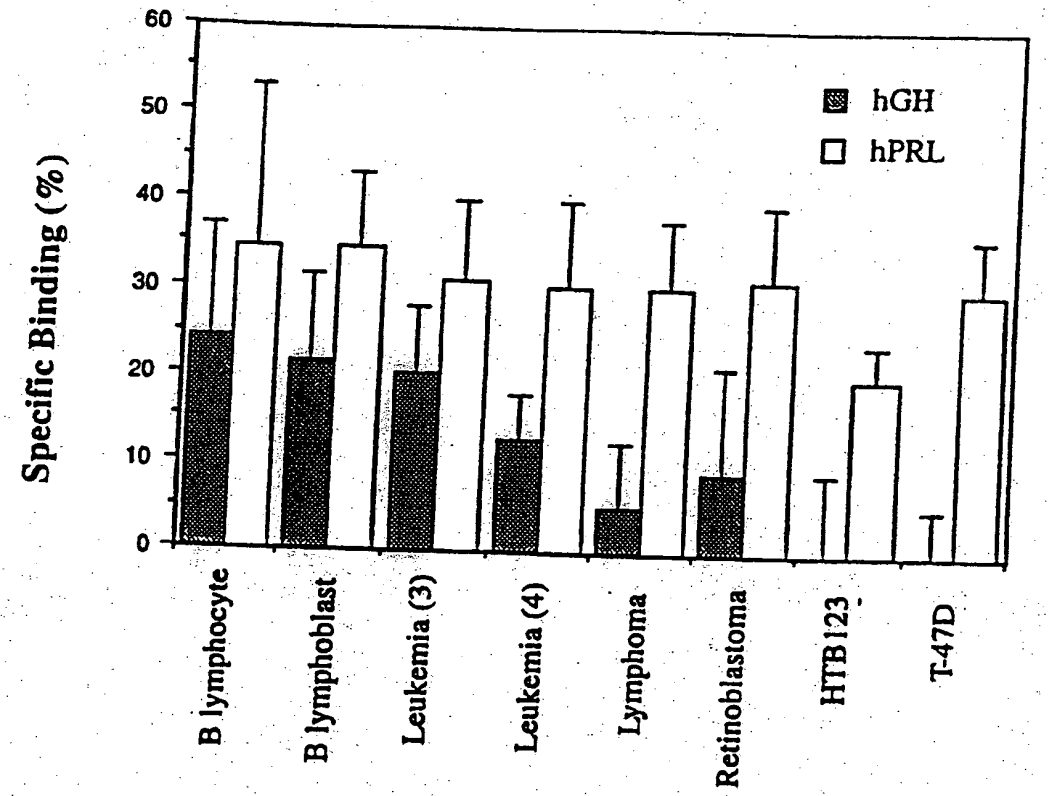

Specific Binding (%)
60
50
40
30
20
10
0
hGH
hPRL
B lymphocyte
B lymphoblast
Leukemia (3)
Leukemia (4)
Lymphoma
Retinoblastoma
HTB123
T-47D

FIGURE 3

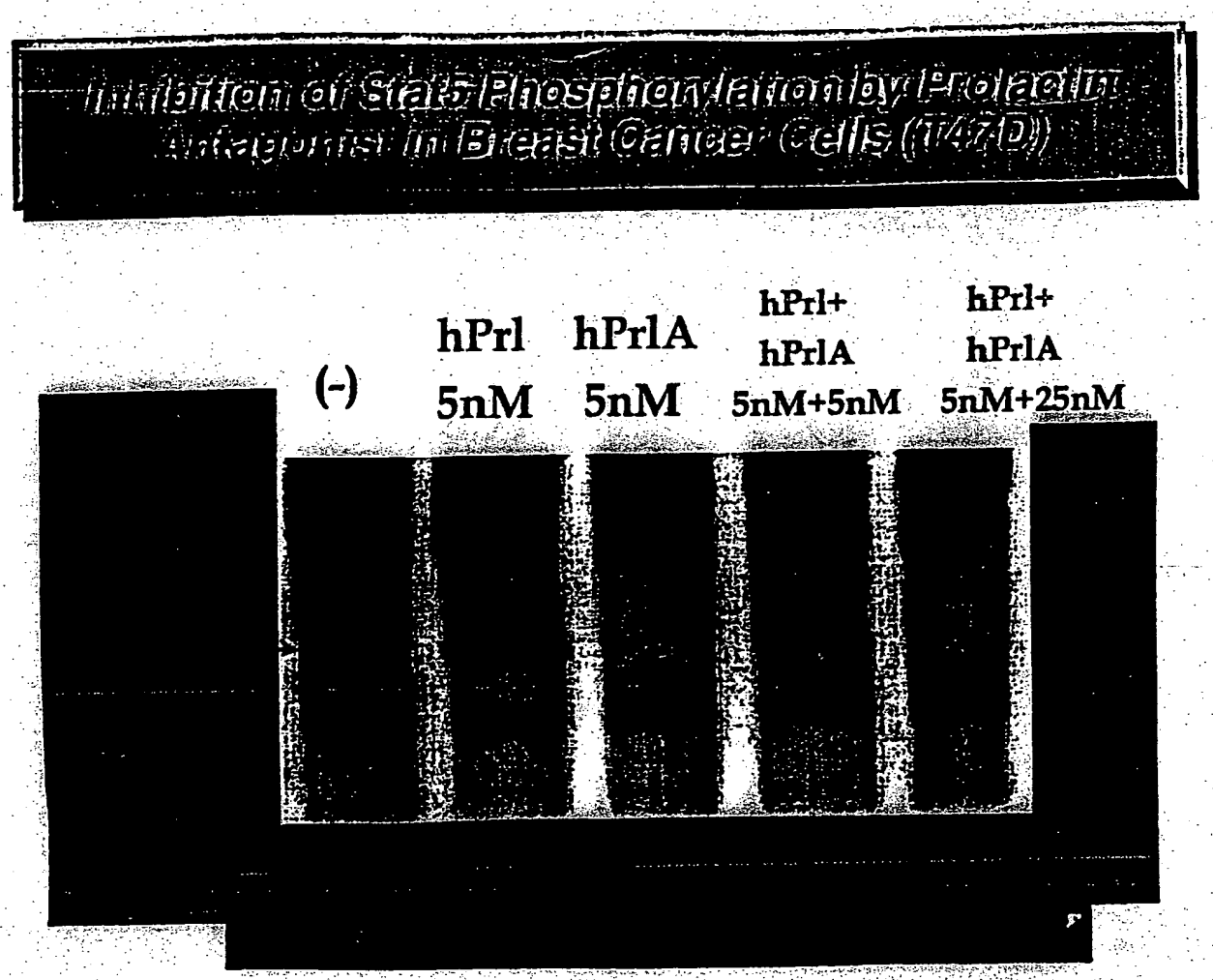

Inhibition of Stat5 Phosphorylation by Prolactin Antagonist in Breast Cancer Cells (T47D)
(-)
hPrl 5nM
hPrlA 5nM
hPrl+ hPrlA 5nM+5nM
hPrl+ hPrlA 5nM+25nM

FIGURE 4

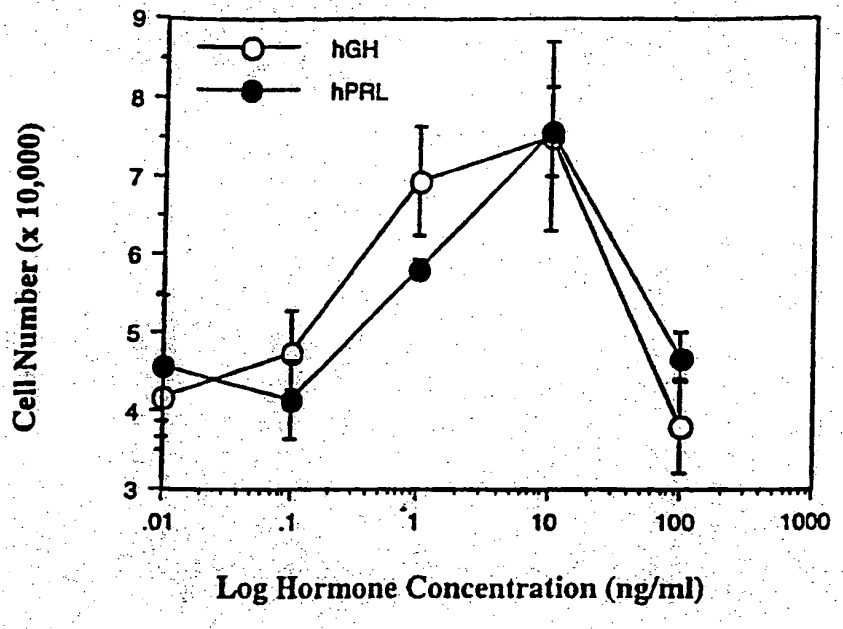
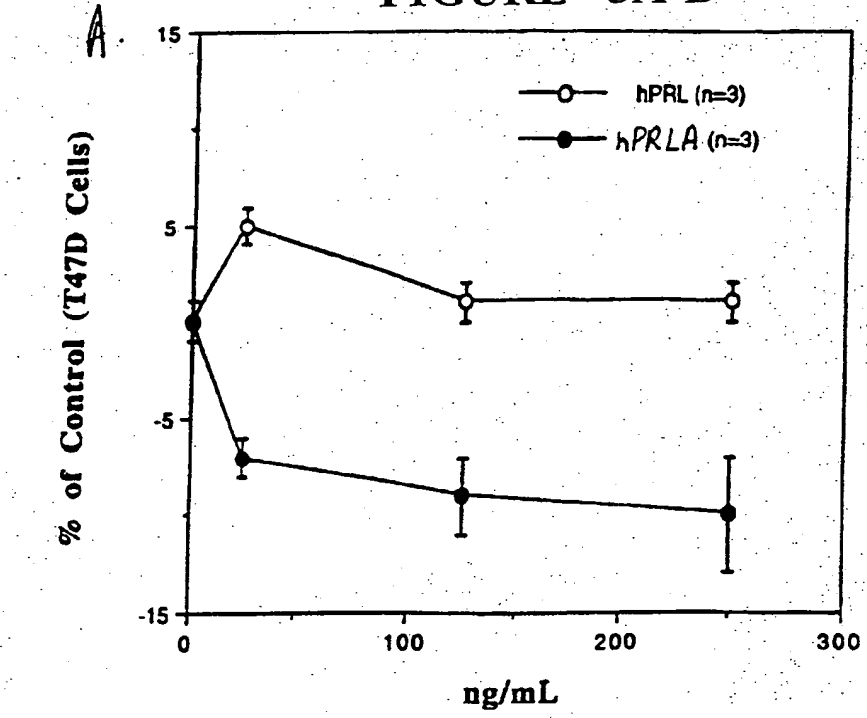

hGH
hPRL
Cell Number (x 10,000)
9
8
7
6
5
4
3
.01
.1
1
10
100
1000
Log Hormone Concentration (ng/ml)

FIGURE 5A-B


A.
hPRL (n=3)
hPRLA (n=3)
% of Control (T47D Cells)
15
5
-5
-15
0
100
200
300
ng/mL

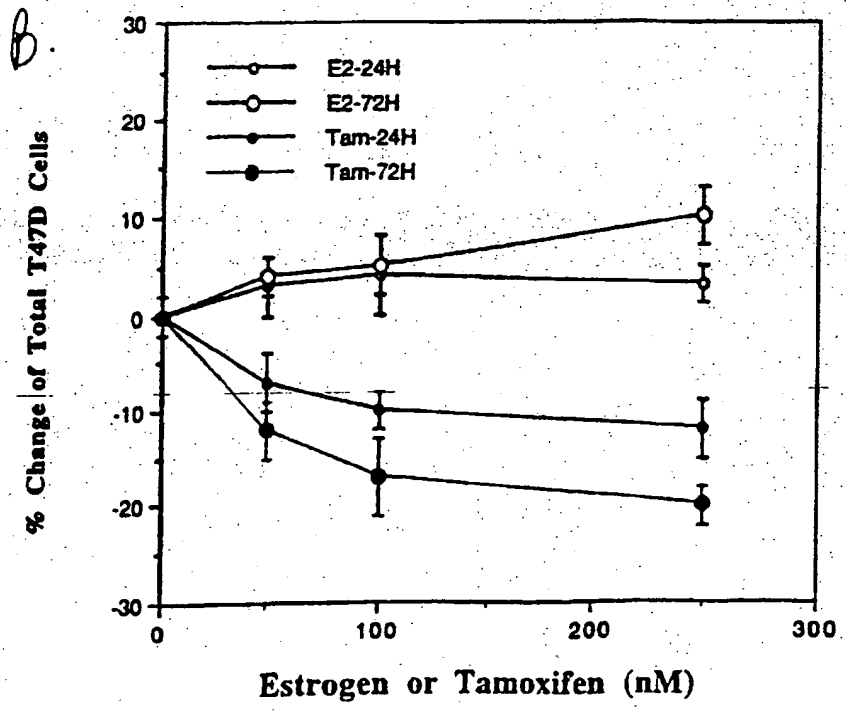

B.
E2-24H
E2-72H
Tam-24H
Tam-72H
% Change of Total T47D Cells
30
20
10
0
-10
-20
-30
0
100
200
300
Estrogen or Tamoxifen (nM)

FIGURE 6

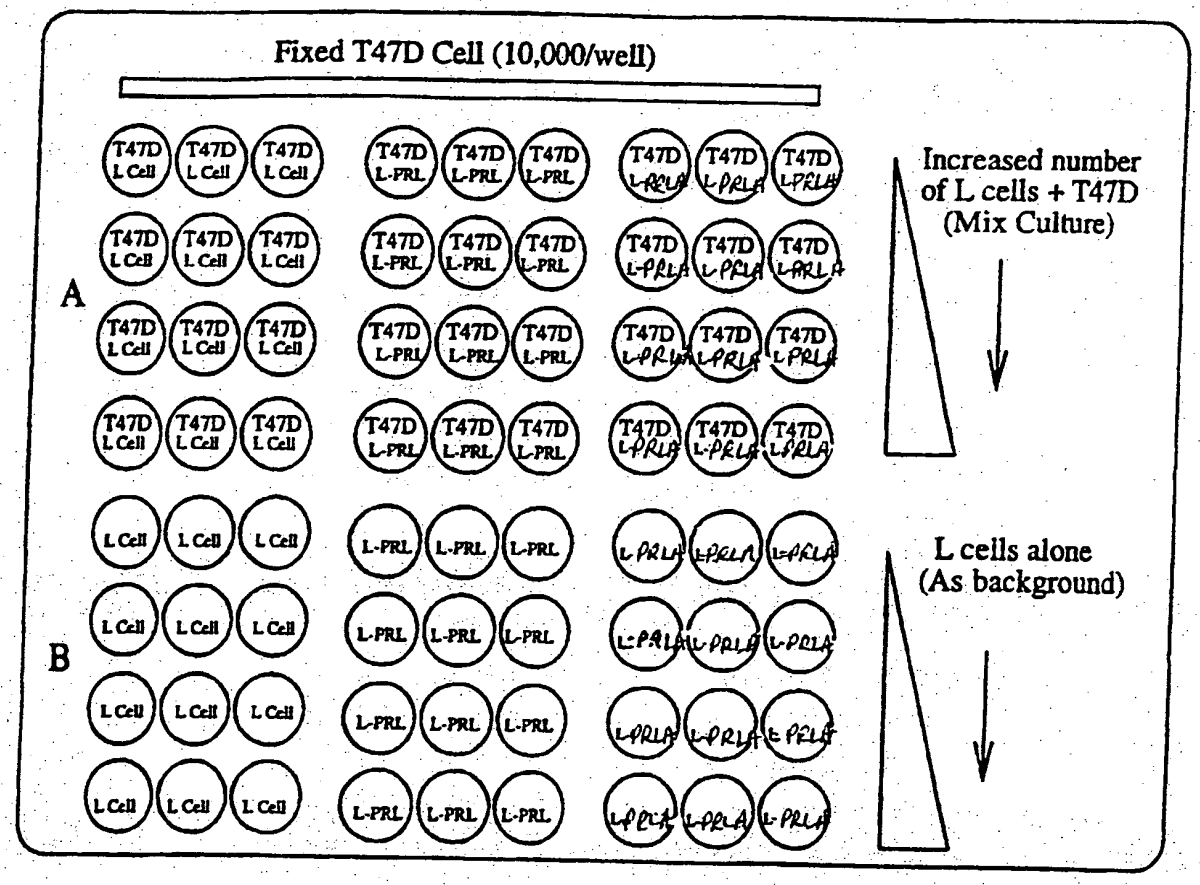
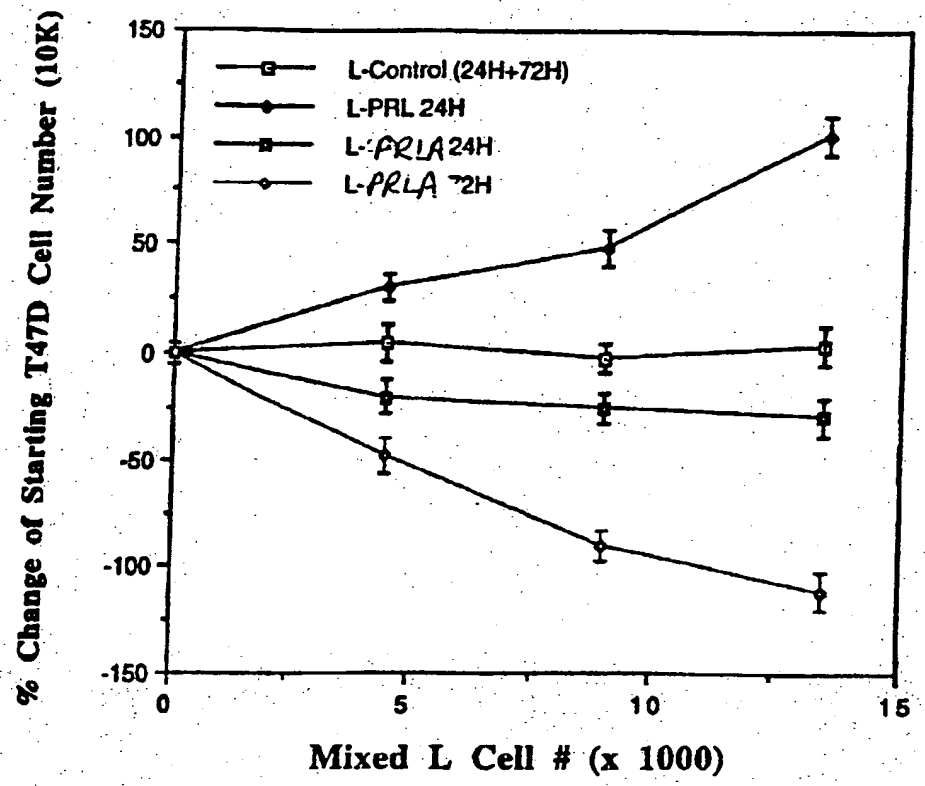

Fixed T47D Cell (10,000/well)
A
B
T47D L Cell
T47D L-PRL
L Cell
L-PRL
Increased number of L cells + T47D (Mix Culture)
L cells alone (As background)

FIGURE 7


% Change of Starting T47D Cell Number (10K)
150
100
50
0
-50
-100
-150
0
5
10
15
Mixed L Cell # (x 1000)
L-Control (24H+72H)
L-PRL 24H
L-PRLA 24H
L-PRLA 72H

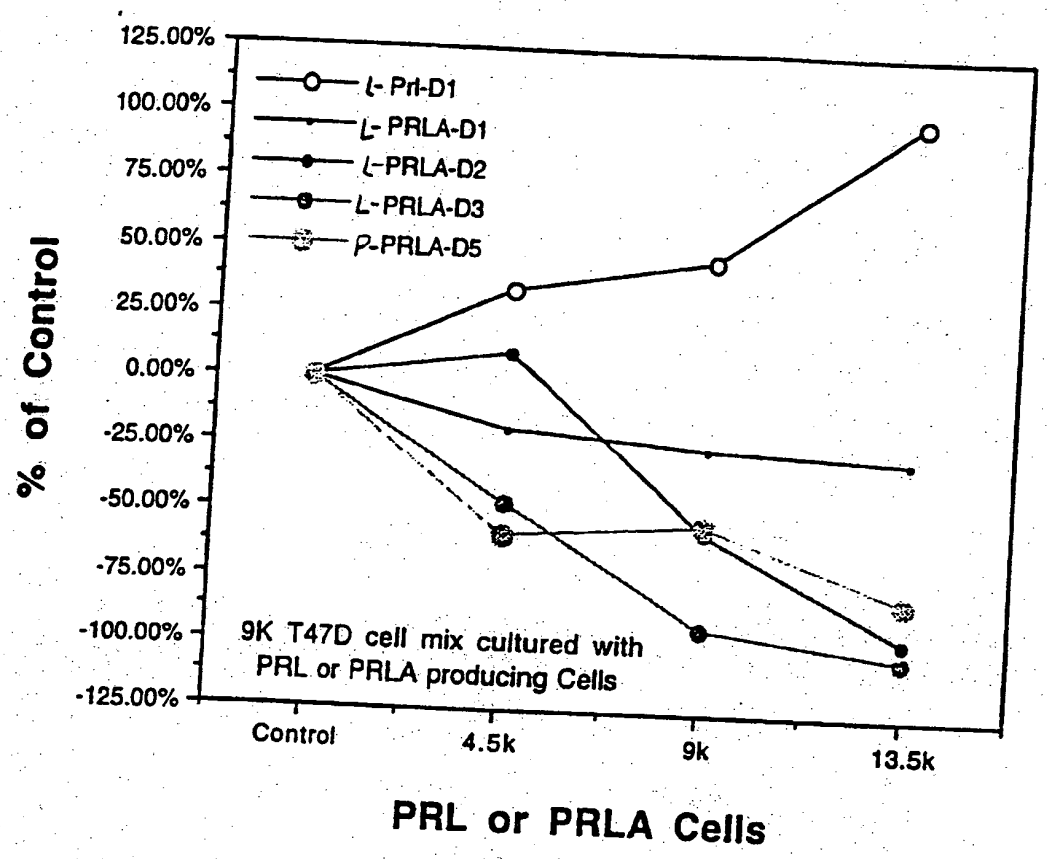
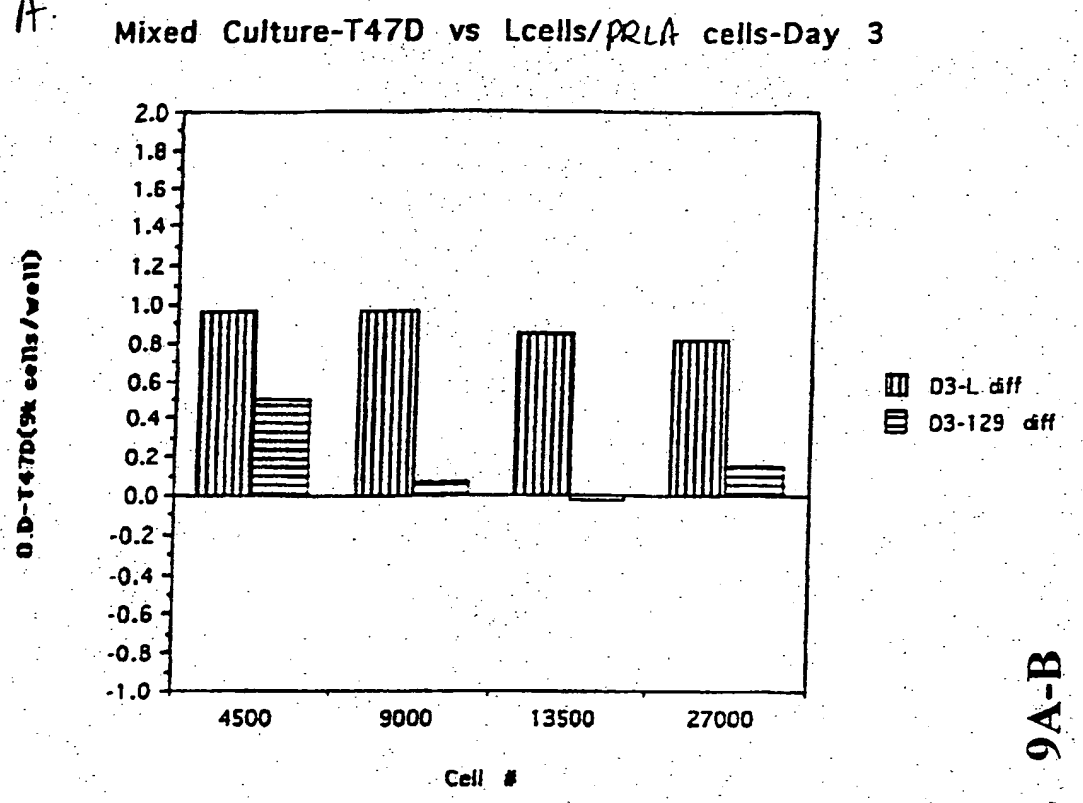

Human Breast Cancer Cell (T47D) Proliferation Assay
125.00%
100.00%
75.00%
50.00%
25.00%
0.00%
-25.00%
-50.00%
-75.00%
-100.00%
-125.00%
% of Control
L- Prl-D1
L- PRLA-D1
L-PRLA-D2
L-PRLA-D3
P-PRLA-D5
9K T47D cell mix cultured with
PRL or PRLA producing Cells
Control
4.5k
9k
13.5k
PRL or PRLA Cells

FIGURE 8

A.



B.

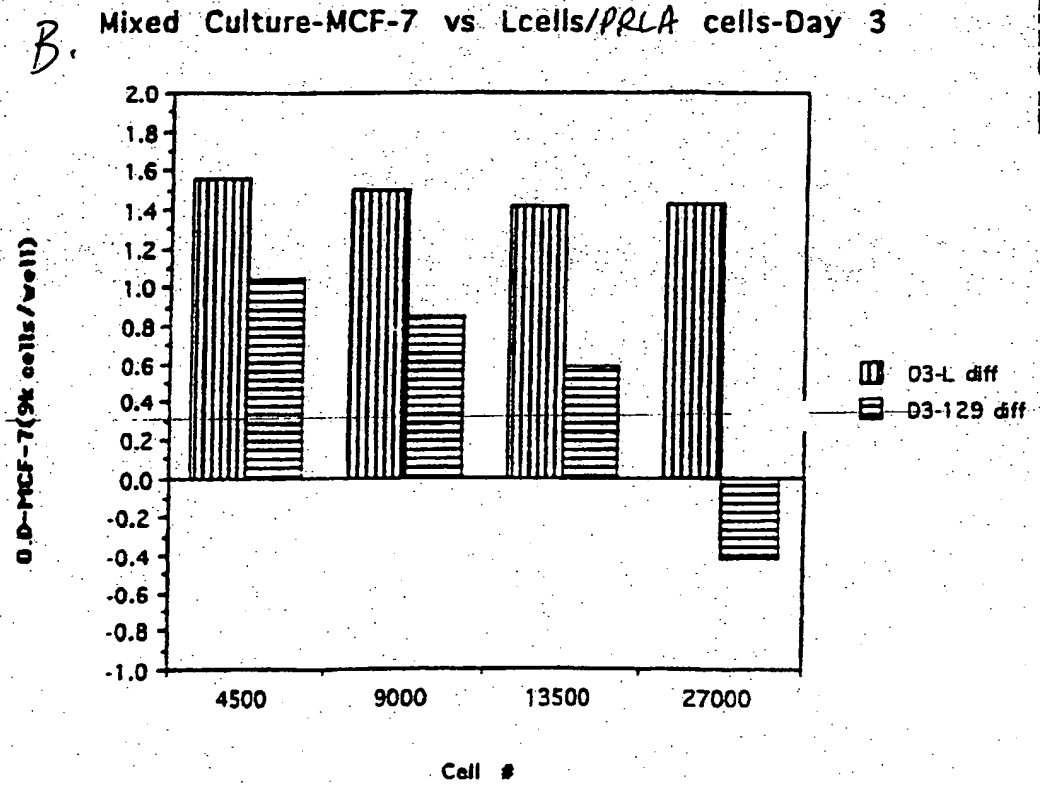


FIGURE 9A-B

FIGURE 10A

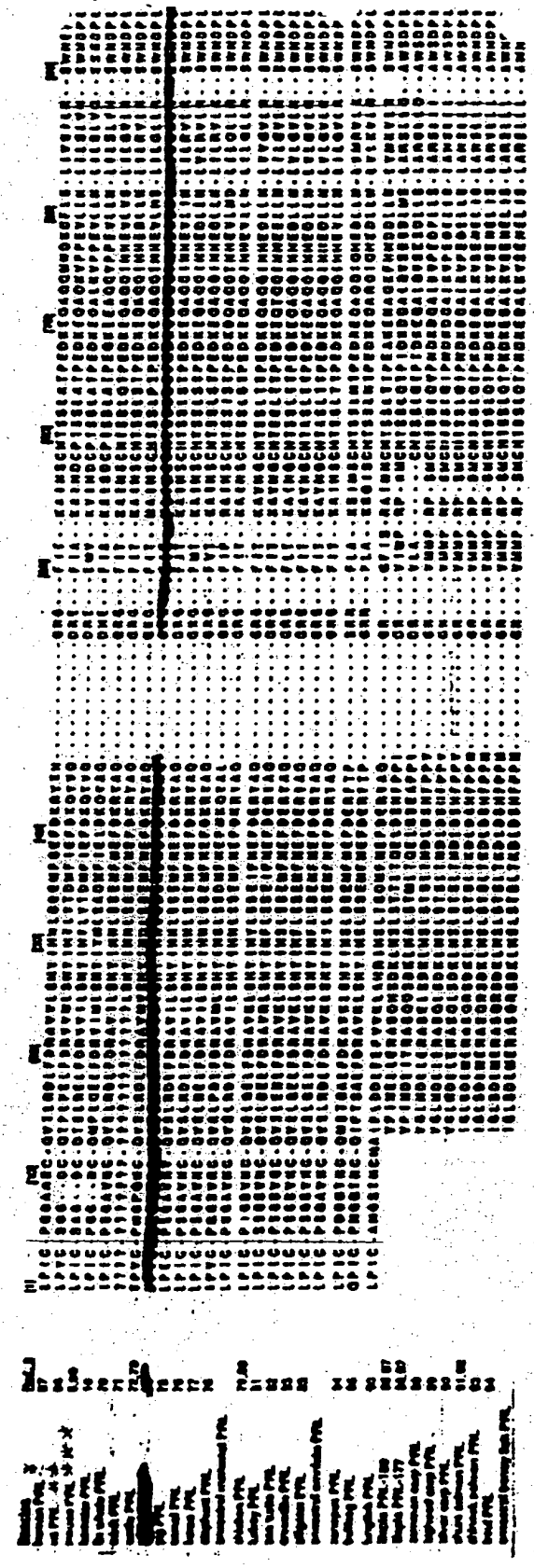


FIGURE 10B

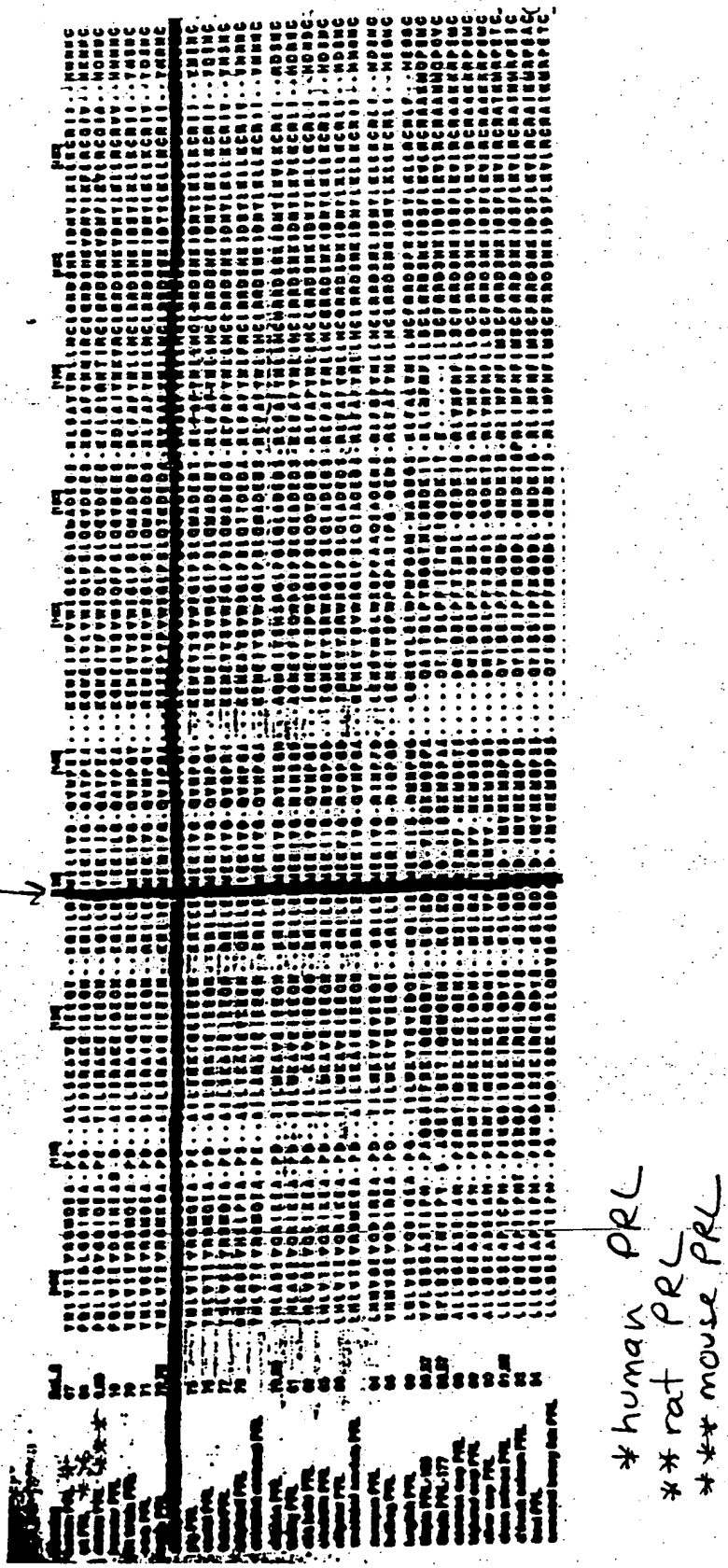


FIGURE 11

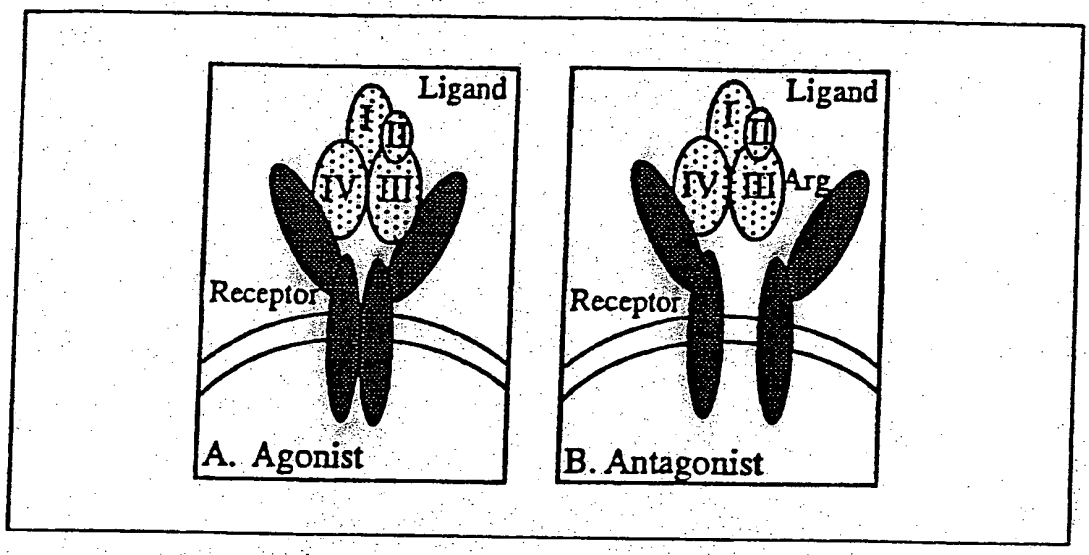

Ligand
I
II
IV
III
Receptor
A. Agonist
Ligand
I
II
IV
III
Arg
Receptor
B. Antagonist

FIGURE 12

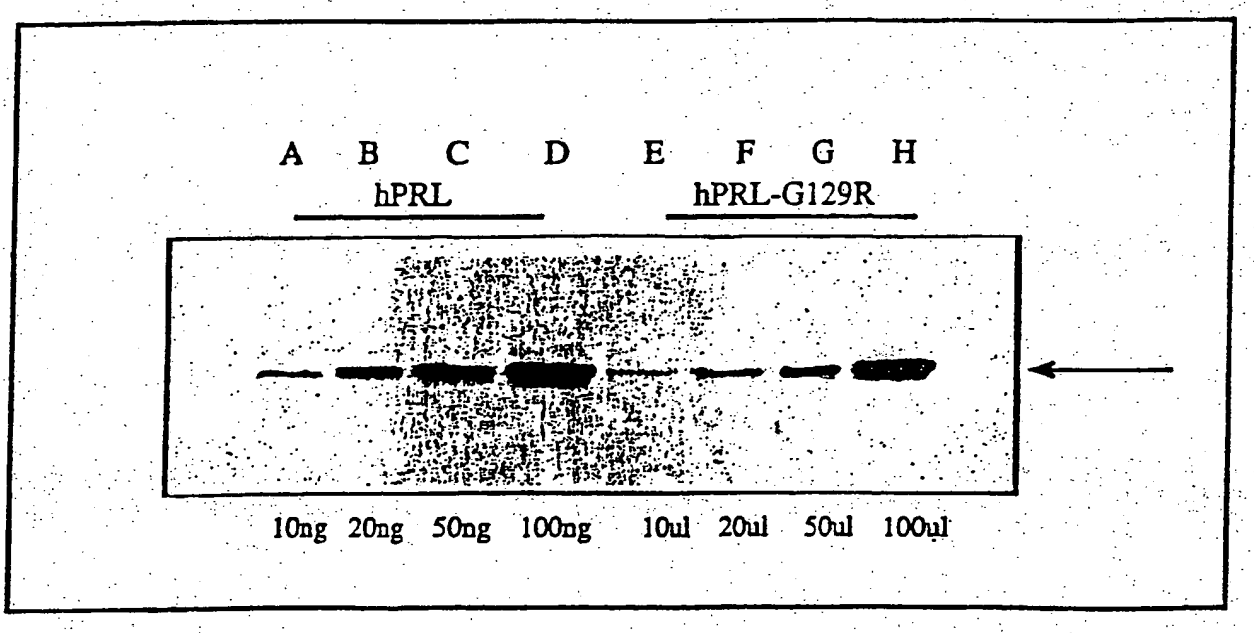

A B C D E F G H
hPRL
hPRL-G129R
10ng 20ng 50ng 100ng 10ul 20ul 50ul 100ul

FIGURE 13

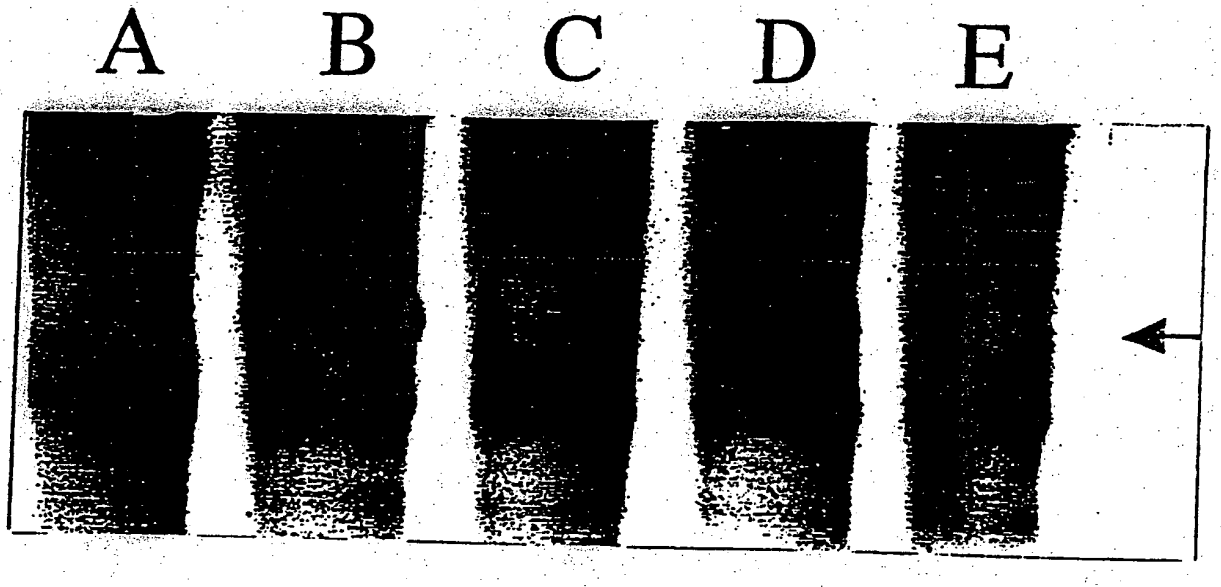
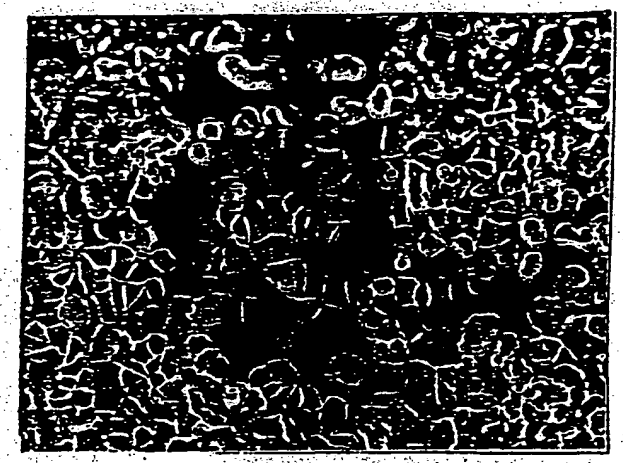
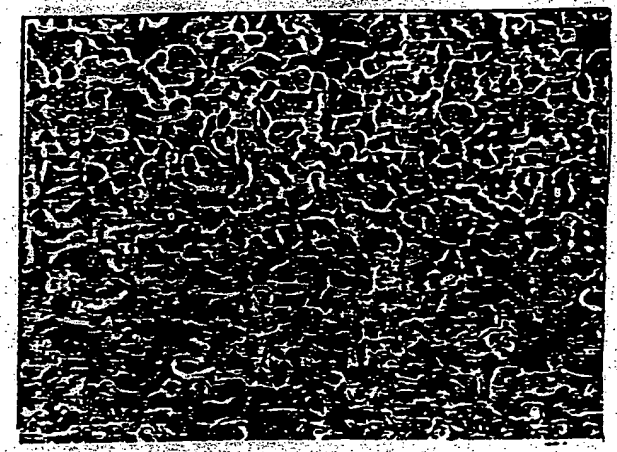


FIGURE 14A-E

A. CONTROL

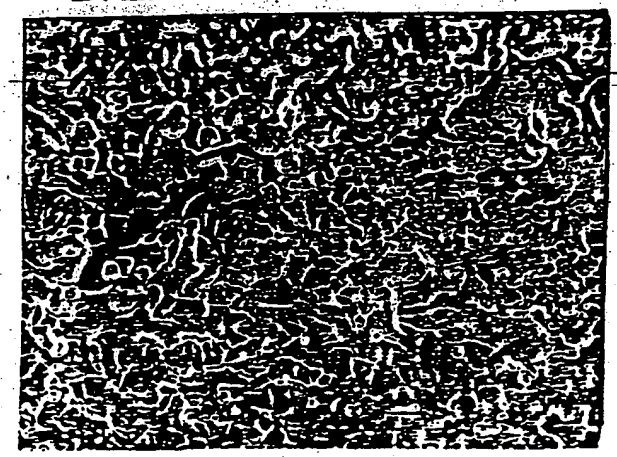


B. hPRL



C. hPRL-G129R

D. E2



E. 4-OH-Tamoxifen

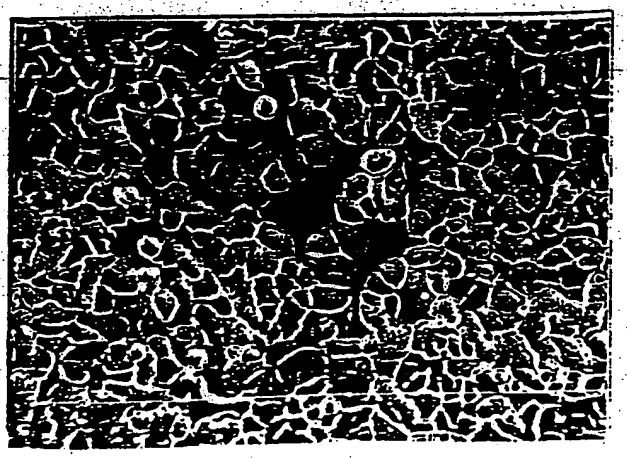


FIGURE 15

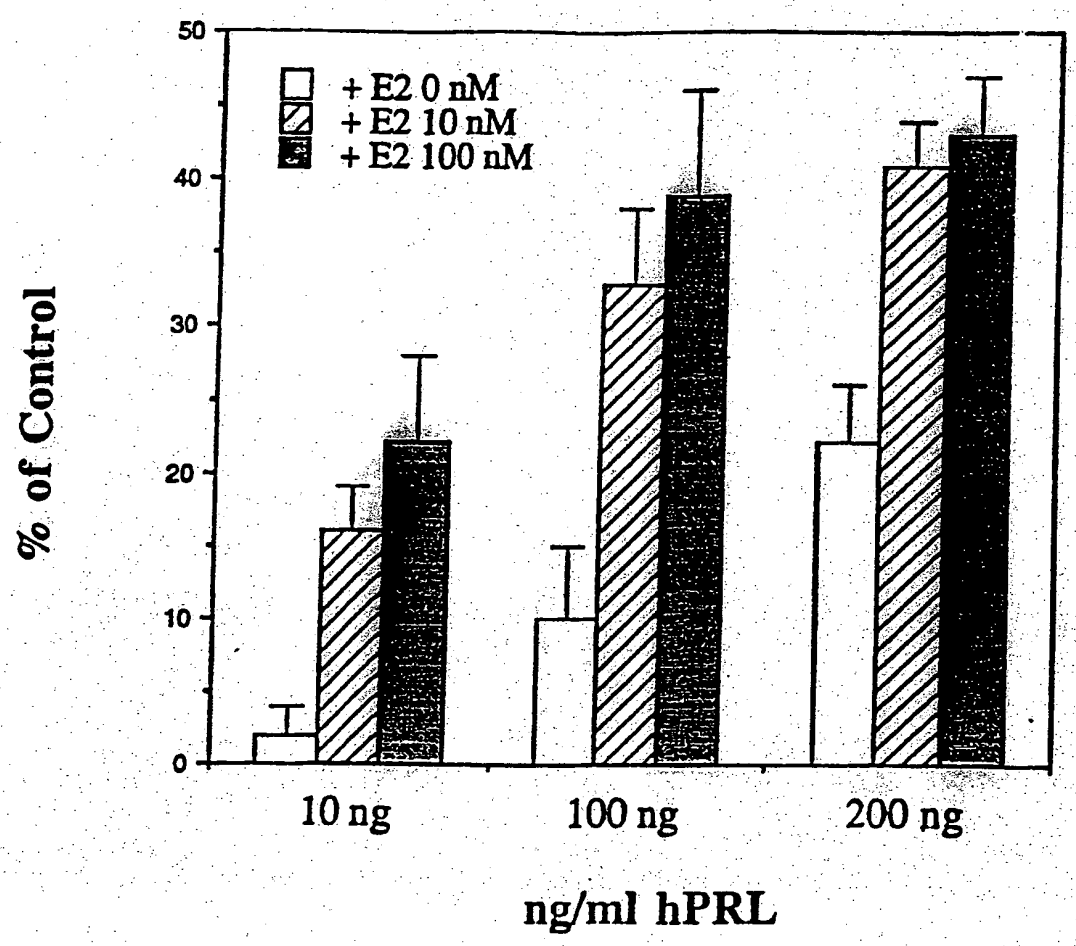
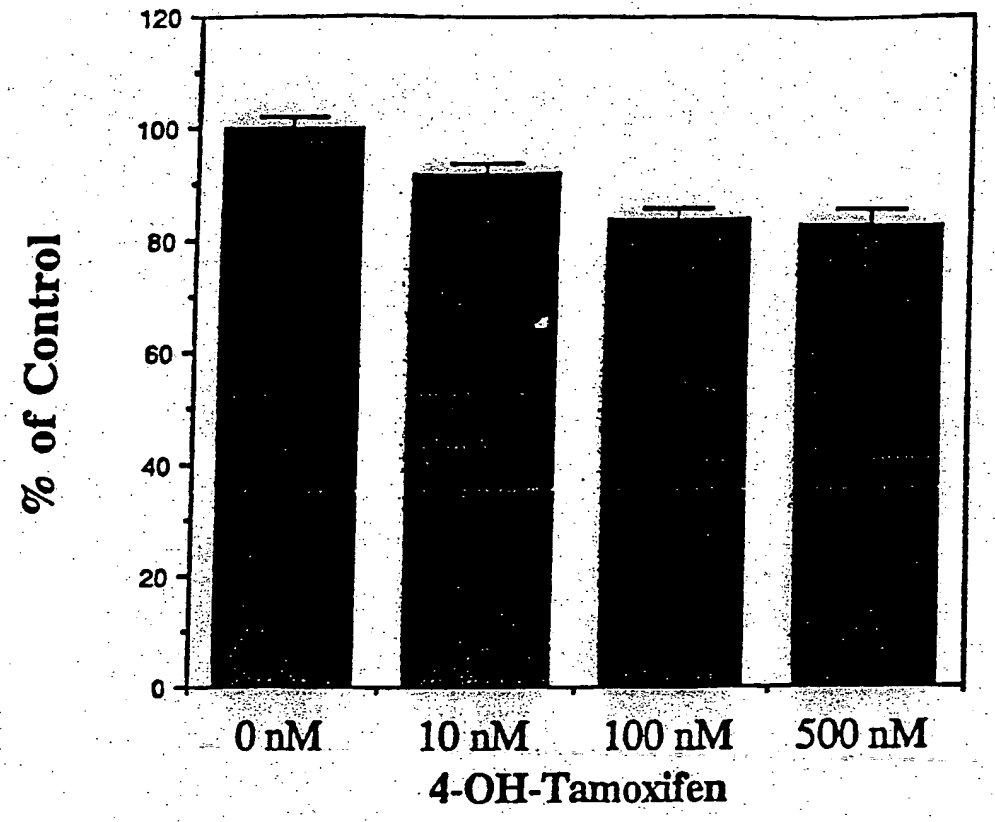

50
40
30
20
10
0
+ E2 0 nM
+ E2 10 nM
+ E2 100 nM
% of Control
10 ng
100 ng
200 ng
ng/ml hPRL

FIGURE 16A-B

A



B

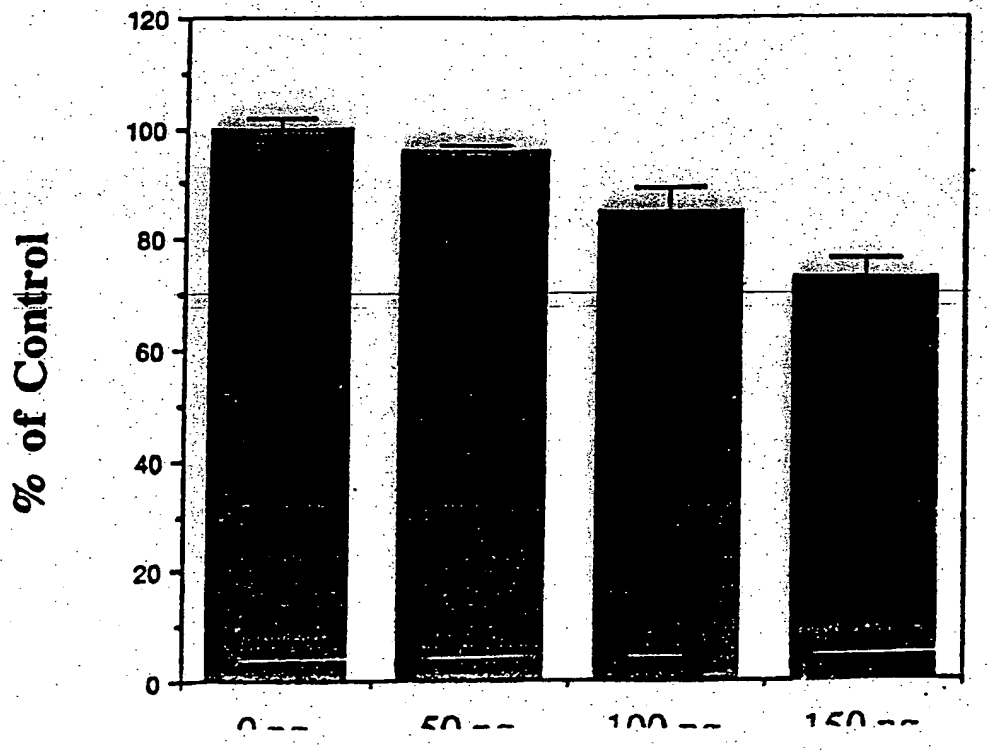


FIGURE 17

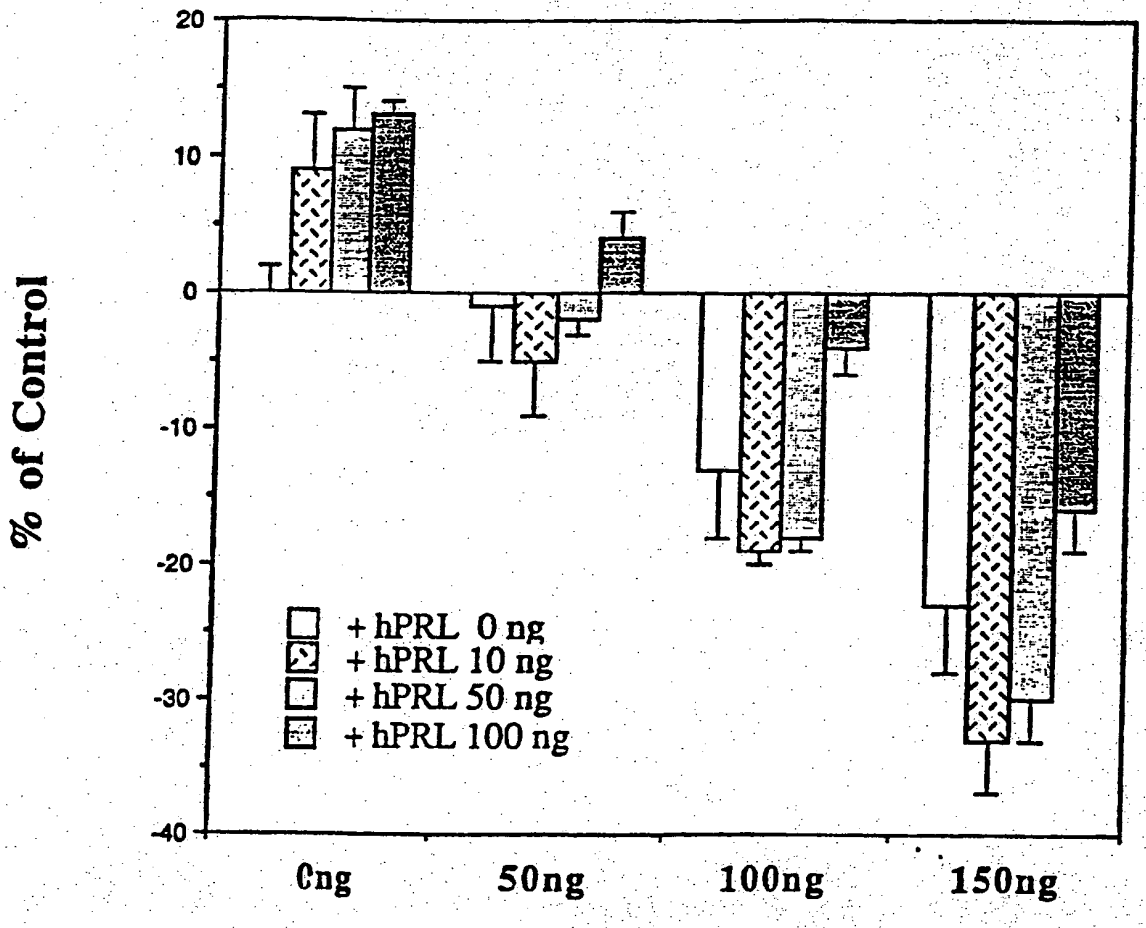
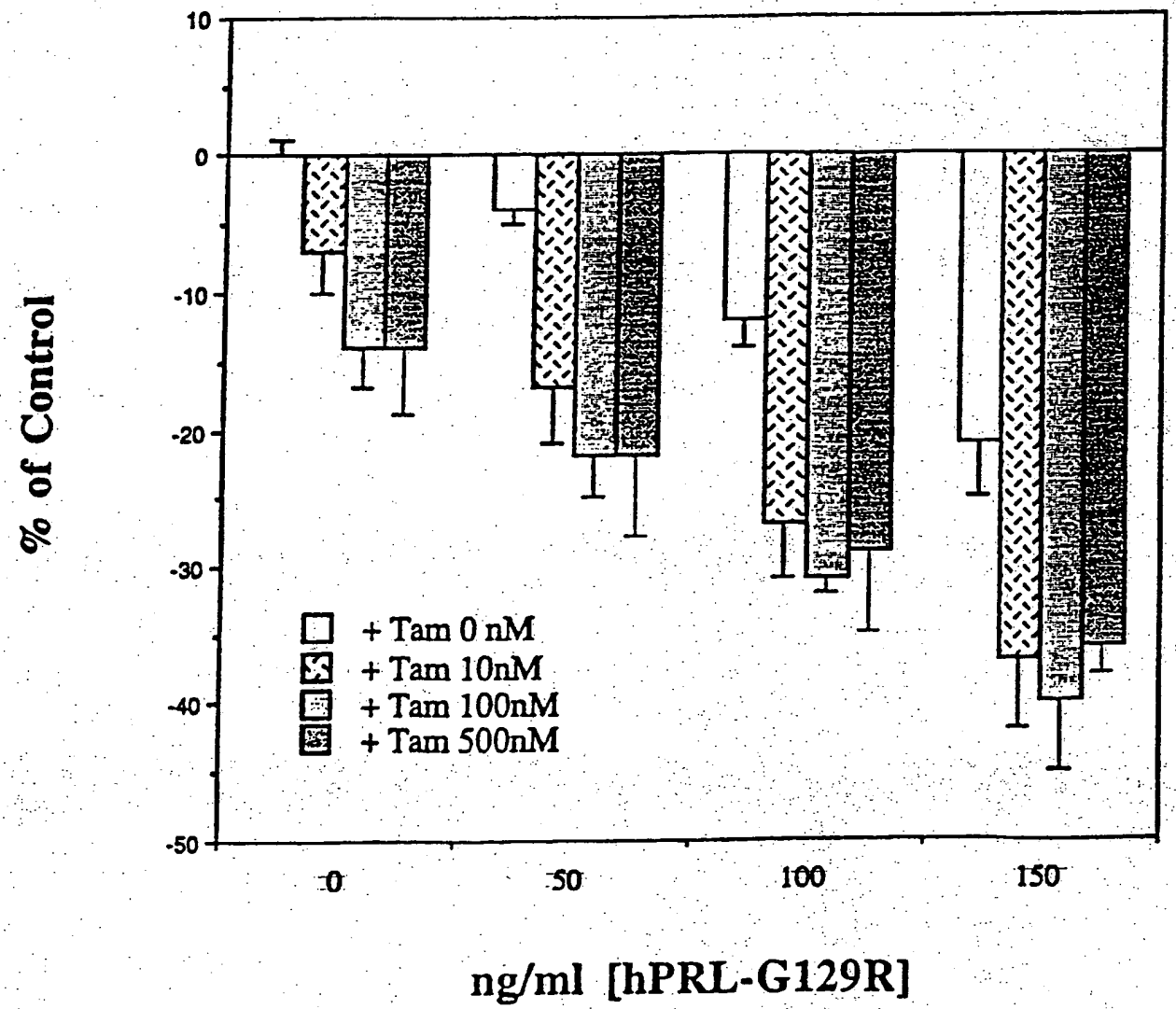
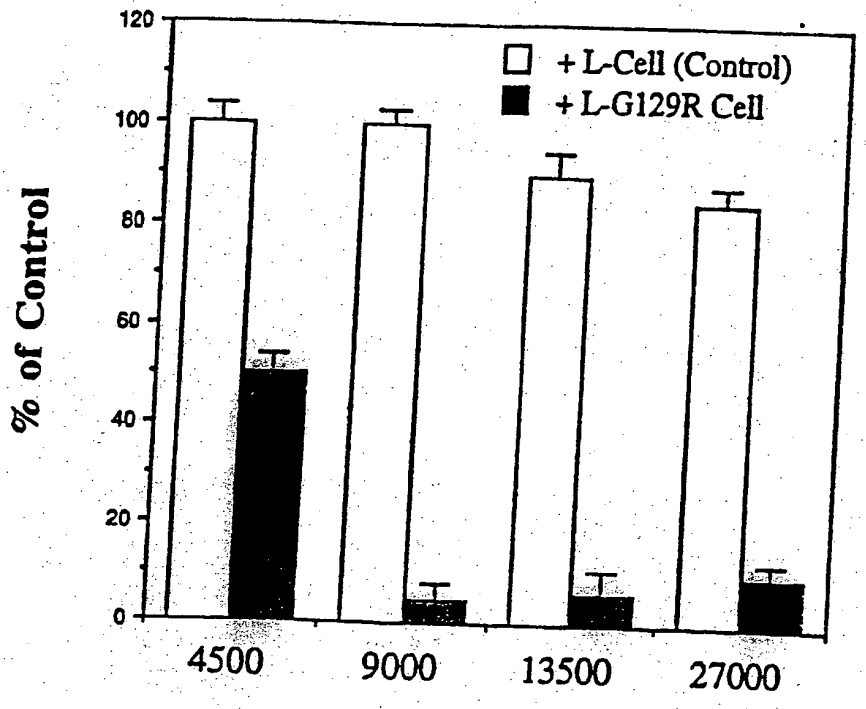

20
10
0
-10
-20
-30
-40
% of Control
+ hPRL 0 ng
+ hPRL 10 ng
+ hPRL 50 ng
+ hPRL 100 ng
Cng
50ng
100ng
150ng
ng /ml [hPRL G129R]

FIGURE 18


10
0
-10
-20
-30
-40
-50
% of Control
+ Tam 0 nM
+ Tam 10nM
+ Tam 100nM
+ Tam 500nM
0
50
100
150
ng/ml [hPRL-G129R]



B. MCF-7 Cells

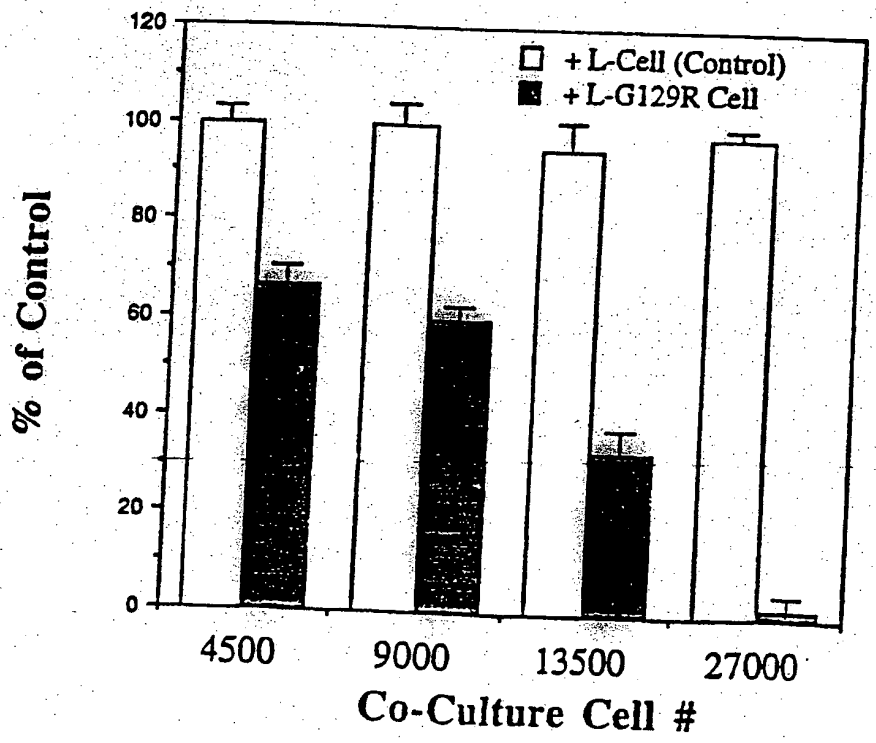


FIGURE 19 A-B

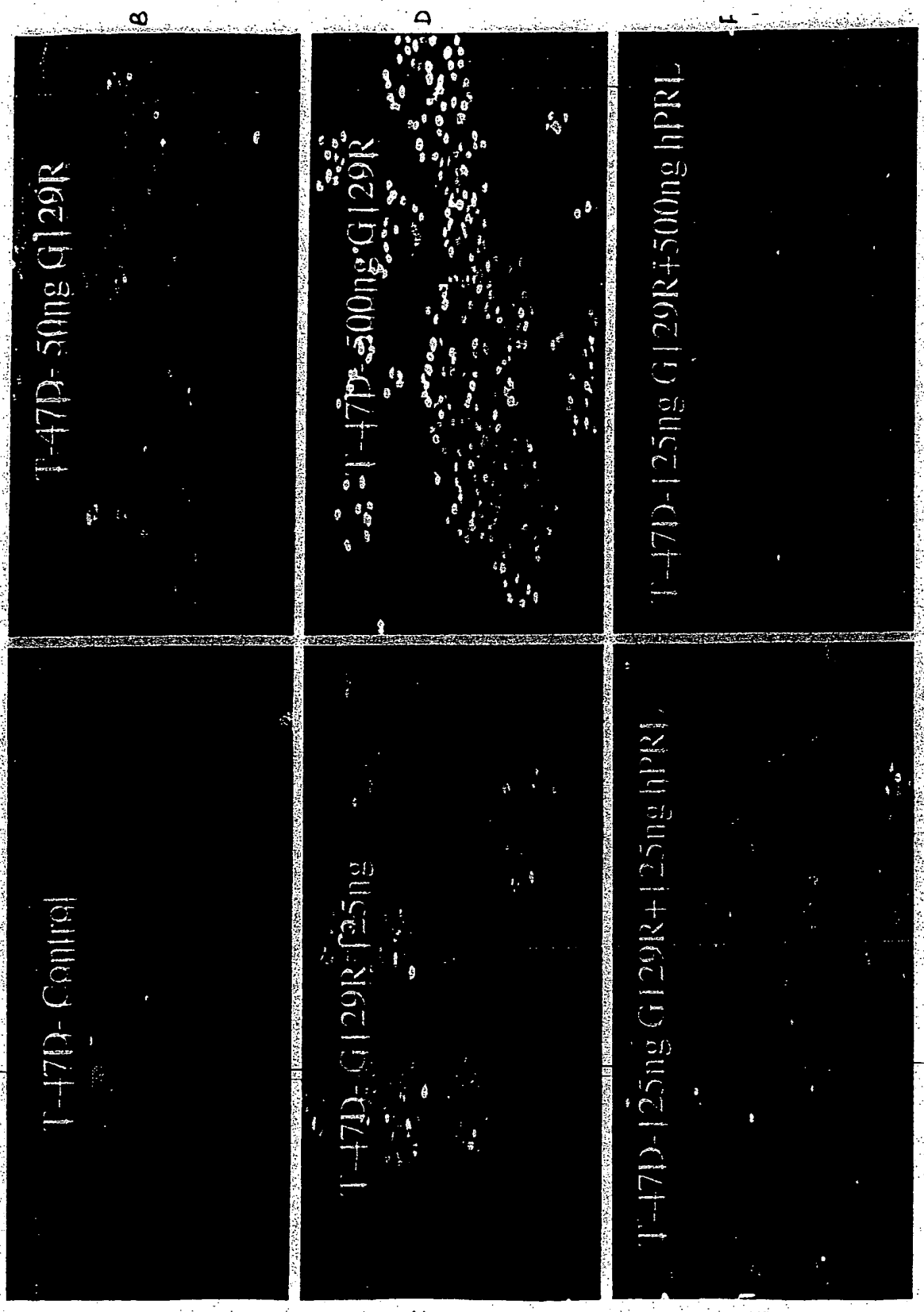

A
T-47D- Control
B
T-47D- 50ng G129R
C
T-47D- G129R-125ng
D
T-47D- 500ng G129R
E
T-47D-125ng G129R+125ng hPRL
F
T-47D-125ng G129R+500ng hPRL

FIGURE 20A-F

A
T-47D- control
B
T-47D- 50ng G129R- 2h
C
T-47D- 50ng G129R- 8h
D
T-47D- 50ng G129R- 24h
E
T-47D- 50ng G129R- 48h

FIGURE 21

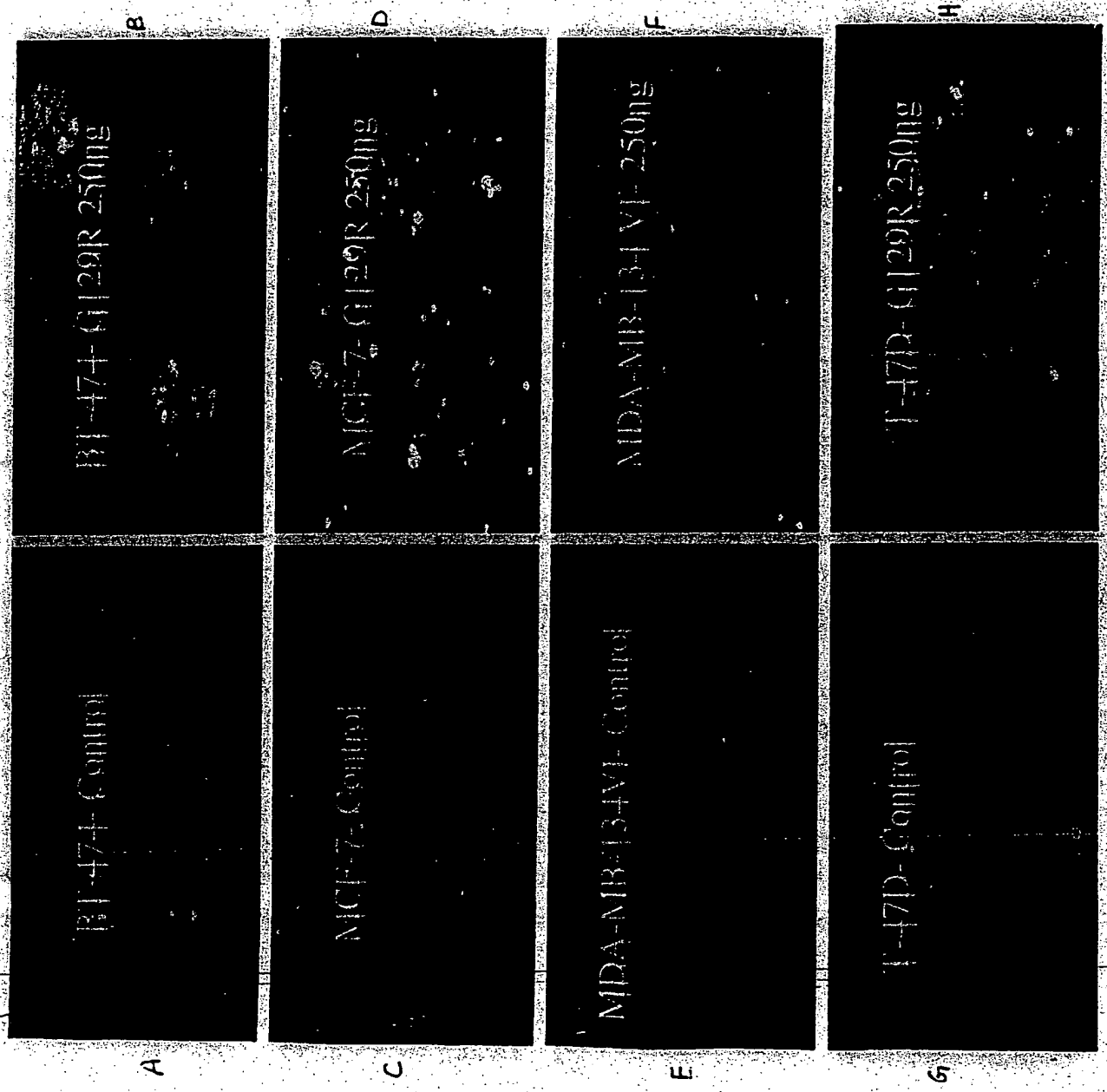

BT-474- Control
BT-474- GH29R 250ng
MCF-7- Control
MCF-7- GH29R 250ng
MDA-MB-134VI- Control
MDA-MB-134VI- 250ng
T-47D- Control
T-47D- GH29R 250ng
A
B
C
D
E
F
G
H

FIGURE 22A-H

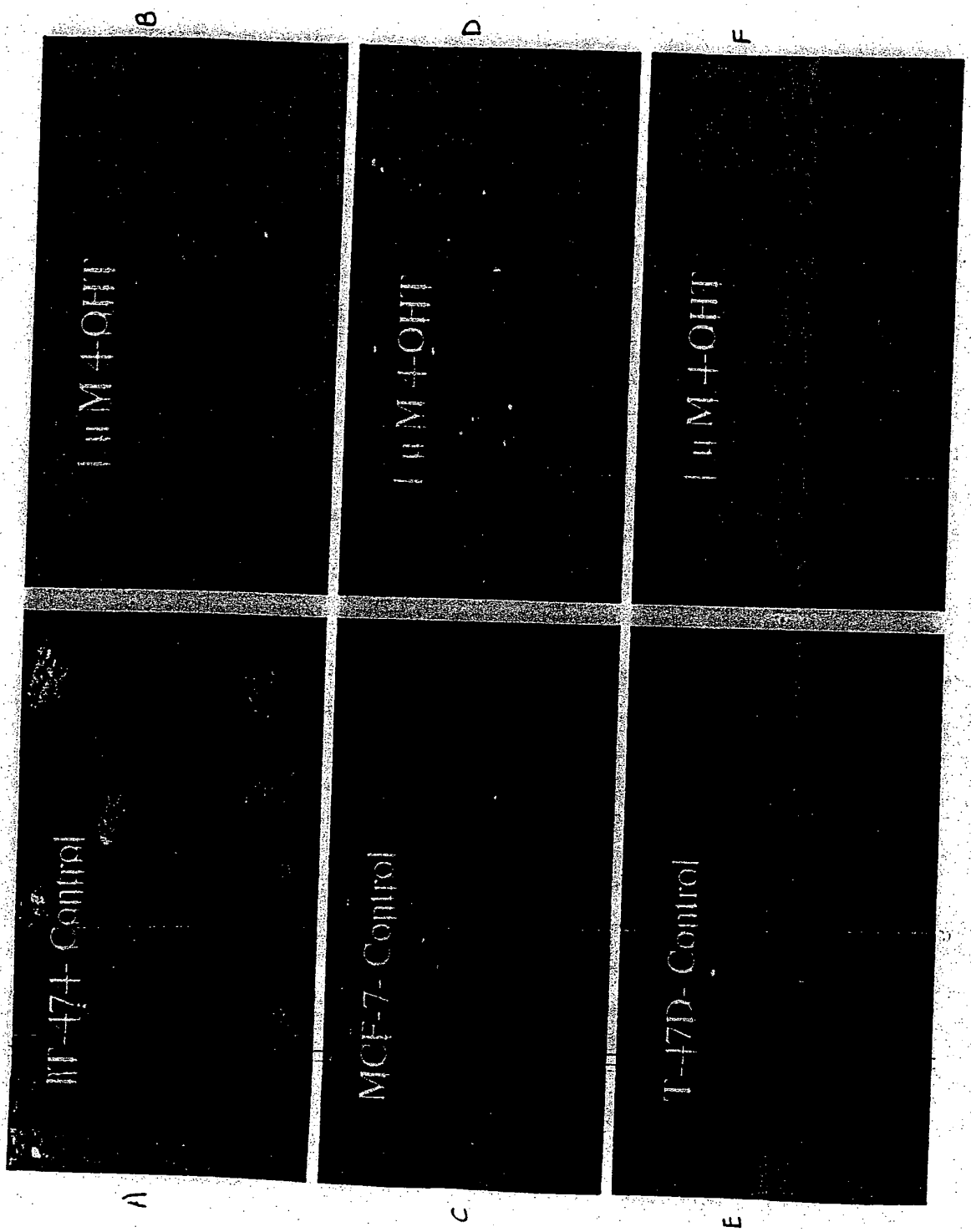

A
MT-47+ Control
B
1 uM 4-OHT
C
MCF-7- Control
D
1 uM 4-OHT
E
T-47D- Control
F
1 uM 4-OHT

FIGURE 23A-F

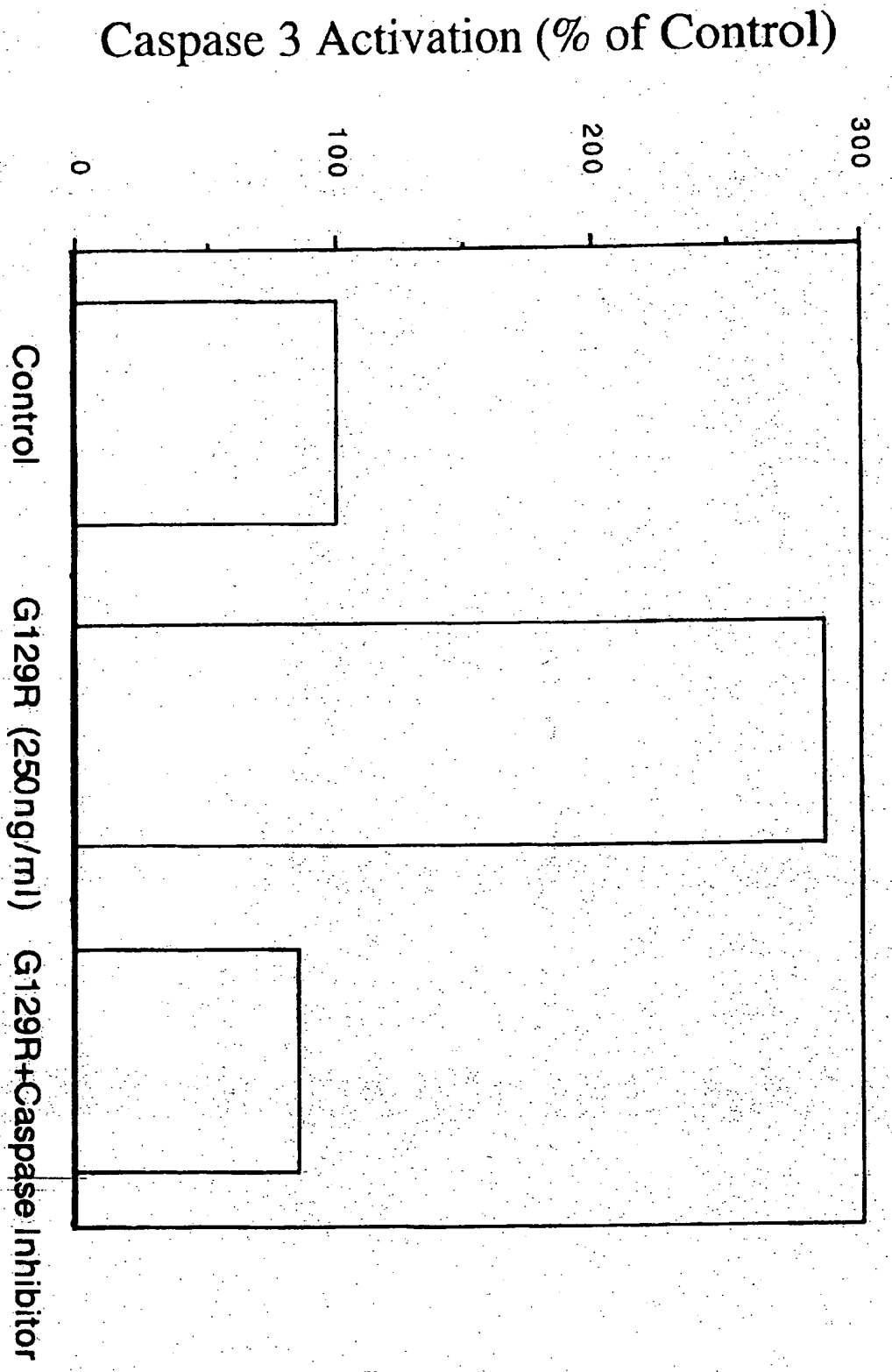

Caspase 3 Activation (% of Control)
0
100
200
300
Control
G129R (250ng/ml)
G129R+Caspase Inhibitor

FIGURE 24

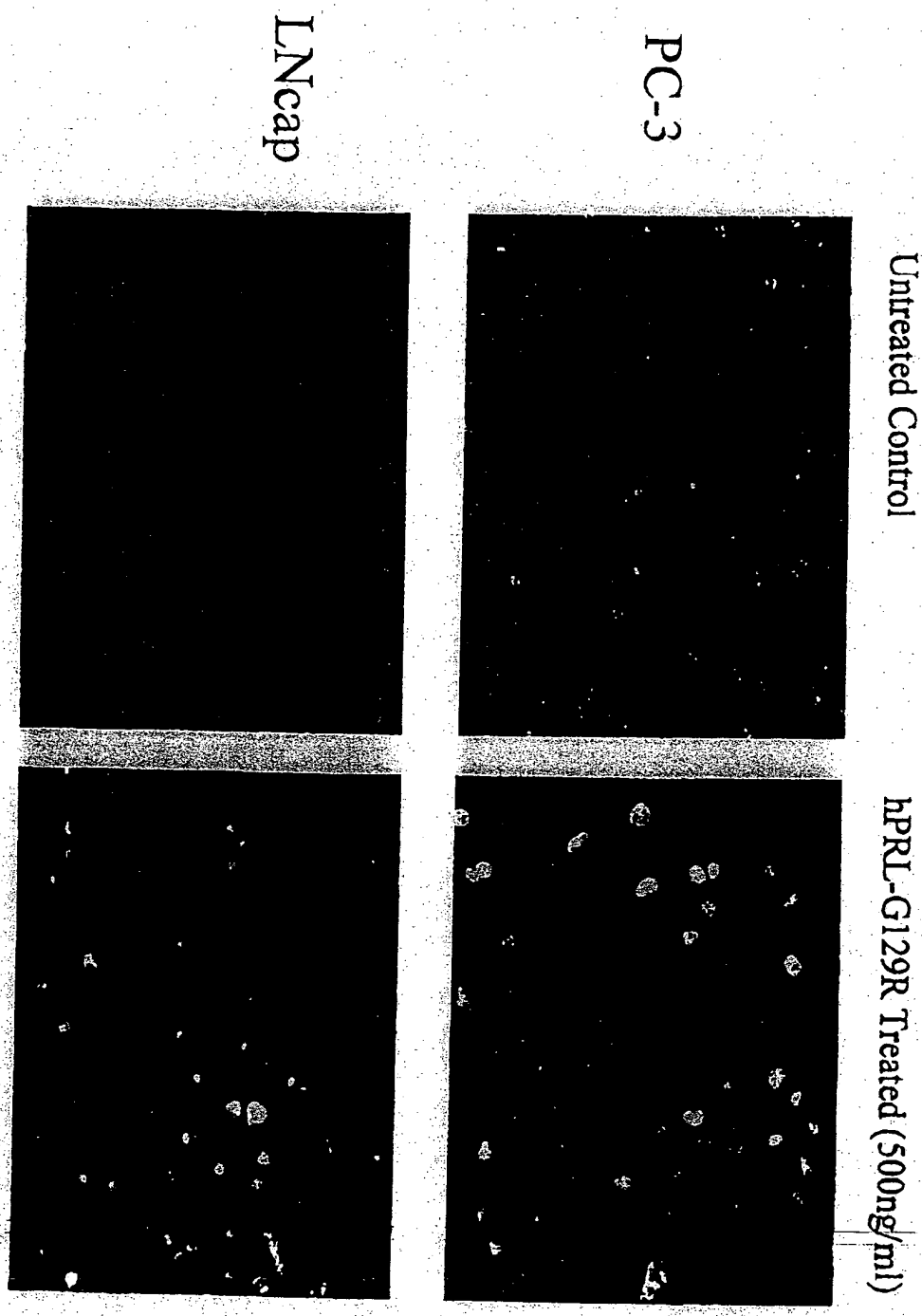


FIGURE 25

SEQUENCE LISTING

<110> CHEN, WEN Y.
WAGNER, THOMAS E.

<120> USE OF ANTI-PROLACTIN AGENTS TO TREAT PROLIFERATIVE CONDITIONS

<130> 31701-PCT

<150> 60/085,128
<151> 1998-05-12

<150> 09/246,041
<151> 1999-02-05

<160> 9

<170> FastSEQ for Windows Version 3.0

<210> 1
<211> 20
<212> DNA
<213> Unknown

<400> 1
gcttagcagt tgttgttgtg 20

<210> 2
<211> 16
<212> DNA
<213> Unknown

<400> 2
atgaacatca aaggat 16

<210> 3
<211> 23
<212> DNA
<213> Unknown

<400> 3
cggctcctag agaggatgga gct 23

<210> 4
<211> 24
<212> DNA
<213> Unknown

<400> 4
gcacttcagt atccatggtc cggt 24

<210> 5
<211> 20
<212> DNA
<213> Unknown

<400> 5
agaaggcagc caacatgaag 20

<210> 6
<211> 20
<212> DNA
<213> Unknown

<400> 6
gcttagcagt tgttgttgtg 20

<210> 7
<211> 16
<212> DNA
<213> Unknown

<400> 7
atgaacatca aaggat 16

<210> 8
<211> 24
<212> DNA
<213> Unknown

<400> 8
cttctagagc gcatggagct cata 24

<210> 9
<211> 23
<212> DNA
<213> Unknown

<400> 9
ccctctagac tcgagcggcc gcc 23

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10232

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : Please See Extra Sheet.
US CL : 424/198.1; 436/63; 514/2, 8; 530/395, 399

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 424/198.1; 436/63; 514/2, 8; 530/395, 399

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, EMBASE, BIOSIS, CAPLUS
search terms: apoptosis, prolactin, prl, hprl, breast, mammary, prostate, cancer, tumor, truncated prolactin receptor

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	BOTENBAL et al. Feasibility, endocrine and anti-tumour effects of a triple endocrine therapy with tamoxifen, a somatostatin analogue and an antiprolactin in post-menopausal metastatic breast cancer: a randomized study with long-term follow-up. British Journal of Cancer. 1998, Vol. 77, No. 1, pages 115-122, especially pages 115, 116 and 118.	1-6
Y	CHEN et al. Development of recombinant human prolactin receptor antagonists by molecular mimicry of the phosphorylated hormone. Endocrinology. 1998, Vol. 139, No. 2, pages 609-616, especially page 615.	1-3

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
27 AUGUST 1999

Date of mailing of the international search report
13 SEP 1999

Name and mailing address of the ISA/US
Commissioner of Patents and Trademarks
Box PCT
Washington, D.C. 20231
Facsimile No. (703) 305-3230

Authorized officer
ANNE L. HOLLERAN
Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10232

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	REYNOLDS et al. Expression of prolactin and its receptor in human breast carcinoma. Endocrinology. 1997, Vol. 138, No. 12, pages 5555-5560, especially page 5560.	1-3
Y	GOFFIN et al. Evidence for a second receptor binding site on human prolactin. Journal of Biological Chemistry. 23 December 1994, Vol. 269, No. 5, pages 32598-32606, especially pages 32599, 32602 and Figure 5B.	1, 2, 7, 8
Y	JANSSEN et al. In vitro characterization of prolactin-induced effects on proliferation in the neoplastic LNCaP, DU145, and PC3 models of the human prostate. Cancer. 01 January 1996, Vol. 77, No. 1, pages 144-149, especially pages 146-148.	7-9
A	NEVALAINEN et al. Prolactin and prolactin receptors are expressed and functioning in human prostate. Journal of Clinical Investigation. February 1997, Vol. 99, No. 4, pages 618-627, see entire document.	7-11, 17-26
A	GOFFIN et al. Antagonistic properties of human prolactin analogs that show paradoxical agonistic activity in the Nb2 bioassay. Journal of Biological Chemistry. 12 July 1996, Vol. 271, No. 22, pages 16573-16579, see entire document.	1-11
A	FUH et al. Mechanism-based design of prolactin receptor antagonists. Journal of Biological Chemistry. 15 March 1993, Vol. 268, No. 8, pages 5376-5381, see entire document.	1-11

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/10232

A. CLASSIFICATION OF SUBJECT MATTER:
IPC (6):

A61K 38/19, 38/27; C07K 14/475, 14/52, 14/61, 14/71, 14/715, 14/72

This Page is Inserted by IFW Indexing and Scanning Operations and is not part of the Official Record

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☑ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☐ FADED TEXT OR DRAWING
- ☑ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☑ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☐ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☐ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.
As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.